The University of Alberta
Printing Department
Edmonton, Alberta

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR : LESLIE L. CARSTENS

TITLE OF THESIS : METABOLITES OF CYATHUS HELENAE

DEGREE FOR WHICH THESIS WAS PRESENTED : M.Sc.

YEAR THIS DEGREE GRANTED : 1974

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

THE UNIVERSITY OF ALBERTA

METABOLITES OF
CYATHUS HELENAE

by

LESLIE L. CARSTENS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL, 1974

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

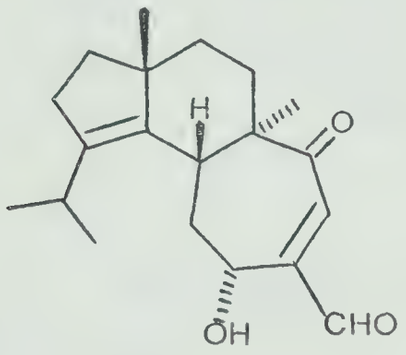
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

METABOLITES OF CYATHUS HELENAE

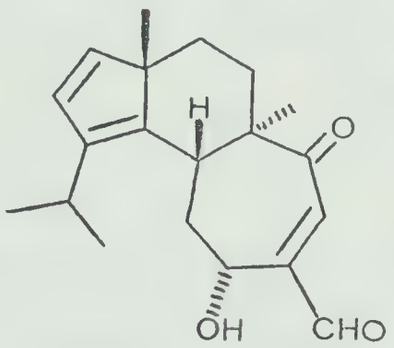
submitted by LESLIE L. CARSTENS in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The recently discovered fungus Cyathus helenae has been shown to produce a number of interesting metabolites including seven diterpenoids and a phenol, 2,4,5-trihydroxybenzaldehyde[4,5]. We herein report the characterization of a previously isolated compound, cyathin B^3 (5) ($C_{20}H_{28}O_3$), and a new compound, which occurs with and crystallizes with cyathin B^3, named cyathin C^3 (6) ($C_{20}H_{26}O_3$).

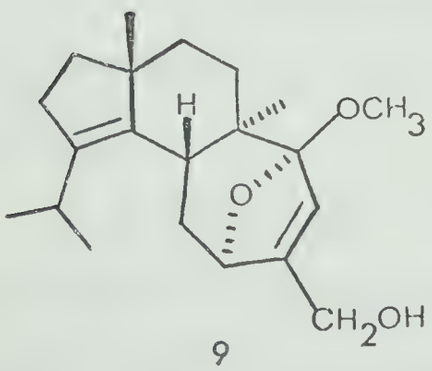


5

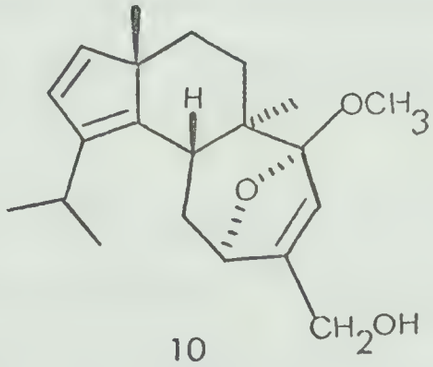


6

The structures of both cyathin B^3 and cyathin C^3 were confirmed through chemical transformations to the known compounds, cyathin A^3 methyl ketal (9), and allocyathin B^3 methyl ketal (10), respectively.



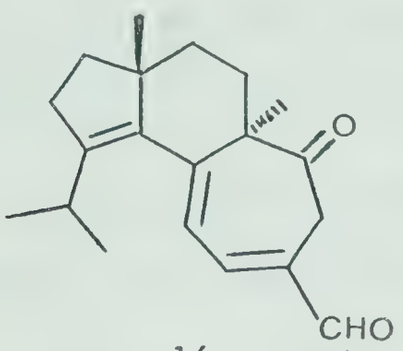
9



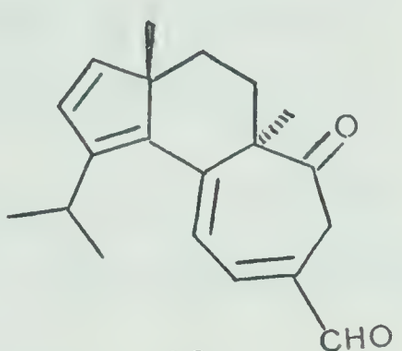
10

The absolute configuration of cyathin A^3 (1) has been determined previously by x-ray crystallography[7].

Acetylation of the cyathin B^3-C^3 mixture gave a mixture of acetates (13) and (14), and the unexpected dehydration products (16) and (18).



16



18

The α,β-unsaturated aldehyde grouping of cyathin B^3-C^3 was found to be very reactive toward nucleophilic Micheal-type reactions. Thus a methanol adduct (20) of cyathin B^3 was prepared and characterized. Selective hydrogenation of the 1,2- and 12,13- double bonds in cyathin B^3-C^3 lead to the C-12 β-epimer of 21 which isomerized to a mixture of C-12 epimers of dihydrocyathin B^3, on silica gel plates.

Also isolated and characterized in this work was a compound identical with the fatty acid, palmitic acid ($C_{16}H_{32}O_2$).

A new compound, cyathin A^2 ($C_{20}H_{30}O_2$), was also isolated and has been partially characterized. Its dehydro analogue, tentatively named cyathin B^2 ($C_{20}H_{28}O_2$) also appears to be produced by the fungus.

ACKNOWLEDGEMENTS

The author wishes to thank the persons who were involved with the research and writing of this thesis:

The National Research Council of Canada and the University of Alberta for financial assistance.

The technical staff members of the Department of Chemistry for the determination of the various spectra.

Dr. H. Taube and the late Dr. A. Allbutt for their assistance and advice in the production and separation of cyathin.

Mrs. J. Ball for typing and graphical work.

His wife, Dana, for encouragement and understanding throughout the research and writing of this thesis.

And especially, Dr. W. A. Ayer for his stimulating character and helpful suggestions in all phases of this project, including the proofing of the manuscript.

TABLE OF CONTENTS

PAGE

I. INTRODUCTION 1

II. RESULTS AND DISCUSSION

Isolation and Structure of Cyathin B^3 and Cyathin C^3

1. Cyathin B^3-C^3 mixture 5
2. Separation of cyathin B^3-C^3 *via* their methyl ketals 19
3. Reduction of cyathin B^3 methyl ketal and cyathin C^3 methyl ketal to known cyathin A^3 methyl ketal and allocyathin B^3 methyl ketal 33

Further Characterization of Cyathin B^3 and Cyathin C^3

1. Initial acetylation experiments 43
2. Anhydrocyathin B^3 and anhydrocyathin C^3 46
3. Michael addition adduct of cyathin B^3 56
4. Hydrogenation of cyathin B^3-C^3 mixture 64

Isolation and Characterization of Palmitic Acid 66

Isolation of Cyathin A^2. 71

Biological Activity of Cyathin B^3-C^3 Mixture . 74

III. GENERAL EXPERIMENTAL

Solvent purity 75

Thin-Layer Chromatography

1. Preparation of thin-layer plates 75

2. Application of sample 76

3. Elution of the plates and detection of the spots or bands 77

4. Recovery of material after preparative tlc 78

5. Recording R_f values and solvent systems 78

Column Chromatography

1. Preparation of the column 79

2. Application of the sample 80

3. Band detection and fraction collecting 80

Measurement and recording of spectra 81

Sample preparation for spectroscopic measurements 82

IV. DETAILED EXPERIMENTAL

Production and isolation of crude cyathin. . 83

Isolation of cyathin B^3-C^3 mixture: Column chromatography 84

Crystallization and characterization of cyathin B^3-C^3 mixture 85

Attempted $AgNO_3$-tlc separation of cyathin B^3-C^3 mixture 85

Ketalization of cyathin B^3-C^3 mixture 86

Argentated silica gel ptlc of cyathin B^3-C^3 methyl ketal mixture. . . . 87

Reduction of cyathin B^3 methyl ketal 88

Reduction of cyathin C^3 methyl ketal 88

Acetylation of cyathin B^3-C^3 mixture 89

Dehydration of cyathin B^3-C^3 *via* acetylation 90

Michael addition to cyathin B^3-C^3 mixture 91

Hydrogenation of cyathin B^3-C^3 mixture 92

Isolation, crystallization, and characterization of palmitic acid 93

Isolation of cyathin A^2 94

V. REFERENCES 96

VI. APPENDIX 98

LIST OF TABLES

TABLE	TITLE	PAGE
I.	NMR Data for cyathin B^3 methyl ketal and cyathin C^3 methyl ketal	26
II.	NMR Data for reduced cyathin B^3 methyl ketal and published cyathin A^3 methyl ketal	37
III.	NMR Data for reduced cyathin C^3 methyl ketal and published allo-cyathin B^3 methyl ketal	41
IV.	NMR Data for anhydrocyathin B^3 and anhydrocyathin C^3	49
V.	NMR Data for methanol adduct of cyathin B^3	61

LIST OF FIGURES

FIGURE	TITLE	PAGE
1	Mass spectrum of cyathin B^3-C^3	9
2	Infrared spectrum (CCl_4) of cyathin B^3-C^3	9
3	Infrared spectrum (nujol mull) of cyathin B^3-C^3	11
4	Nuclear magnetic resonance spectrum (CCl_4) of cyathin B^3-C^3	14
5	Nuclear magnetic resonance spectrum (CCl_4 + trace CF_3COOH) of cyathin B^3-C^3	14
6	Mass spectrum of cyathin B^3 methyl ketal	24
7	Infrared spectrum (CCl_4) of cyathin B^3 methyl ketal	24
8	Mass spectrum of cyathin C^3 methyl ketal	28
9	Infrared spectrum (CCl_4) of cyathin C^3 methyl ketal	28
10	Proton designations for cyathin B^3 and cyathin C^3 methyl ketals	30
11	Mass spectrum of reduced cyathin B^3 methyl ketal	36
12	Infrared spectrum (CCl_4) of reduced cyathin B^3 methyl ketal (upper) and compared to published cyathin A^3 methyl ketal infrared spectrum (lower)	36
13	Mass spectrum of reduced cyathin C^3 methyl ketal	40

14	Infrared spectrum (CCl_4) of reduced cyathin C^3 methyl ketal (upper) and compared to published allocyathin B^3 methyl ketal infrared spectrum (lower)	40
15	Mass spectrum of anhydrocyathin B^3	48
16	Infrared spectrum (CCl_4) of anhydrocyathin B^3	48
17	Mass spectrum of anhydrocyathin C^3	55
18	Infrared spectrum (nujol mull) of anhydrocyathin C^3	55
19	Mass spectrum of methanol adduct cyathin B^3	59
20	Infrared spectrum (CCl_4) of methanol adduct of cyathin B^3	59
21	Mass spectrum of isolated palmitic acid	68
22	Infrared spectrum (CCl_4) of isolated palmitic acid	68
23	Mass spectrum of cyathin A^2	73
24	Infrared spectrum (CCl_4) of cyathin A^2	73
25	Nuclear magnetic resonance spectrum ($CDCl_3$) of cyathin A^2	73

LIST OF SCHEMES

SCHEME	TITLE	PAGE
I	Keto-alcohol, hemi-ketal equilibrium in cyathin B^3-C^3	12
II	Proposed scheme to correlate cyathin B^3-C^3 with known compounds	19

I. INTRODUCTION

In 1965, a new species of fungus, growing in the Canadian Rockies, was discovered by H. J. Brodie[1]. He classified this new bird's nest fungus as belonging to the class Basidiomycetes, subclass Homobasidiomycetes, order Gasteromycetes, family Nidulariaceae, genus Cyathus, and named it Cyathus helenae. This fungus was given the number 1500 and only cultures of this strain were used in the work presented in this thesis.

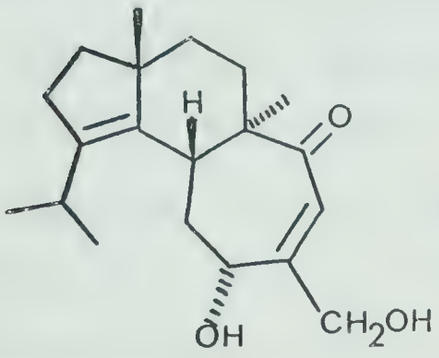
Early work showed that this fungus could be grown on liquid culture and that it showed anti-bacterial antagonism to contaminates[2]. At this time the name "cyathin" was given to the substance(s) responsible for the antibacterial activity.

Johri[3] found that an ethyl acetate extract of the fungal broth (grown on liquid media) retained the antibacterial activity. This method of extraction has been used in all of the work conducted on this organism.

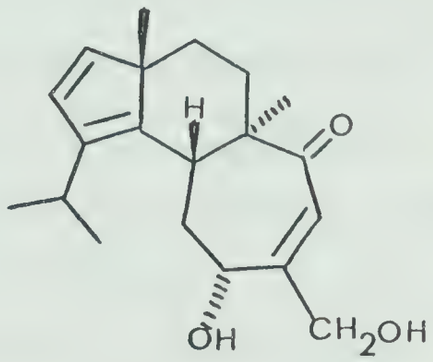
Previous work on the chemistry of the crude cyathin has been carried out in this department, and had led to the characterization of six diterpenoid components, along with the phenol 2,4,5-trihydroxybenzaldehyde (also called chromocyathin)[4]. Among the diterpenoid constituents isolated were cyathin B[3] and cyathin A[3]. The cyathins are named by a simple method. The number of hydrogens in the molecular formula gives the letter designation: 30 = A series, 28 = B series, etc. The number of oxygens is

denoted by the superscript. Isomers of previously isolated compounds are placed in the allo series, for example: cyathin B^3 is $C_{20}H_{28}O_3$ and allocyathin B^3 is also $C_{20}H_{28}O_3$.

Later work by Hubert Taube of this department led to the crystallization and structure elucidation of cyathin A^3 (1) and a new member, allocyathin B^3 (2)[5,6].



1



2

The stereochemistry depicted, including the absolute configuration has been confirmed by x-ray diffraction methods[7]. It was anticipated that the remaining diterpenoid constituents would have the same carbon skeleton as in 1 and 2.

When the work presented in this thesis was initiated, the objective was to determine and prove the structures of cyathin B^3 and other metabolites appearing in the same region on thin-layer chromatograms (see the General Experimental for thin-layer methods).

The fungal metabolites used in this work were prepared by the author. A stock culture of the fungus

was kept on agar petri plates and agar slant tubes. The agar medium (Brodie medium) contained the following: dextrose, 2.0 g; maltose, 5.0 g; yeast extract, 2.0 g; asparagine, 0.2 g; peptone, 0.2 g; glycerol, 6.0 ml; KH_2PO_4, 0.5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $MgSO_4$, 0.24 g; $Fe_2(SO_4)_3$, trace; agar, 20 g; distilled water, 1 l.

After a good growth had appeared (2 - 3 weeks), the agar plates were used to innoculate a number of 2 l Fernbach flasks containing 500 ml of sterile defined medium. This medium contained the following reagents per liter of distilled water: dextrose, 30 g; asparagine, 1.5 g; KH_2PO_4, 1.0 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $MgSO_4$, 0.24 g; $ZnSO_4 \cdot 7H_2O$, 364 μg; thiamine·HCl, 150 μg. Innoculation was accomplished by the addition of 4 - 8 discs taken from the agar plates.

In the latter part of this work, an innoculum flask (500 ml), containing a 3 - 4 week growth of fungus on defined medium was used to innoculate the Fernbach flasks. This was accomplished by the use of a sterile Waring blender to break up the mycelium small enough so that a sterile pipet could be used for the transfer.

By using either innoculation method, the fungus was allowed to grow for 25 - 30 days, at which time it was harvested by either straining the mycelium from the broth with the aid of cheesecloth, or by simply pouring off the broth and leaving the mycelium behind. This latter harvesting method allowed regeneration of growth by simply

adding fresh, sterile medium to the flasks.

The fungal broth was then extracted with an equal volume of ethyl acetate (twice, with half volumes each time). Drying and evaporation of the solvent under reduced pressure gave a yellow-brown foam, called crude cyathin. Chromatographic separations were then carried out on this material as explained in the next chapter.

II. RESULTS AND DISCUSSION

Isolation and Structure of Cyathin B^3 and Cyathin C^3

1) Cyathin B^3-C^3 Mixture

Thin-layer chromatography (tlc) on silica gel has proven very effective in identifying the cyathin constituents. During previous work in these laboratories a compound of R_f(A) 0.60 was characterized by high resolution mass spectrometry as having the molecular formula $C_{20}H_{28}O_3$ and was given the name cyathin B^3 [4,5]. (See the General Experimental, p 78 for the method of reporting R_f values and solvent systems.) When a small amount of electronic phosphor is used with the adsorbant and the developed and air dried plate is viewed under uv light (254 nm), the spot corresponding to cyathin B^3 shows as a dark area on a green background. This method is useful when the compound to be detected has a chromophore absorbing around 250 nm (ref 8, p 20), and also has the advantage of being non-destructive. The spot corresponding to cyathin B^3 may also be detected by spraying the plate lightly with 30% H_2SO_4 and subsequently heating to 100°. This latter method produces a dark purple spot after a few minutes of heating, which gradually turns black.

Since preparative thin-layer chromatography (ptlc) proved very laborious and expensive for large scale preparations, column chromatography was used to separate the cyathin compounds. Silicic acid (Mallinkrodt,

100 mesh) was found to be the best adsorbant; alumina gave very poor results as previously reported[5]. An adsorbant to substrate ratio of 40 : 1 had previously been employed to separate the crude cyathin[4,5]. When this was doubled to 80 : 1, better resolution of the band corresponding to cyathin B^3 was observed. Using a quartz column and with 1% electronic phosphor in the adsorbant, the cyathin B^3 band could be detected and collected when eluted from the column. In fact, using chloroform (0.75% ethanol stabilizer) cyathin B^3 was the only major component eluted from the column. The other cyathin compounds (A^3, A^4, chromocyathin, etc.) could be eluted from the column by grading up to 5% methanol in the chloroform solvent.

On one occasion in the early part of this work column chromatography of a cyathin B^3 rich metabolite mixture gave, much to our delight, crystalline material after removal of the solvent. Recrystallization was accomplished with ether-pentane (soluble ether, insoluble pentane). A second recrystallization produced colorless needles, m.p. 131 - 133°.

High resolution mass spectrometry indicated the molecular formula $C_{20}H_{28}O_3$ for the apparent parent peak at m/e 316, thus identifying this crystalline material as cyathin B^3. The mass spectrum (figure 1) also shows a peak at m/e 314 of 11% intensity. At this point an analogy was drawn with the case of cyathin A^3 - allocyathin B^3 previously studied in these laboratories, in which both com-

pounds occurred together on both column and thin-layer chromatography. It was therefore tentatively assumed that the peak at m/e 314 in the mass spectrum of cyathin B^3 was due to another compound, a dehydro analogue of cyathin B^3. This crystalline material will be referred to as cyathin B^3-C^3 mixture. The following spectral data will confirm this assumption and suggest structures for both cyathin B^3 and cyathin C^3.

Further comparison of the cyathin B^3-C^3 mass spectrum with those of cyathin A^3 and allocyathin B^3 revealed a similarity in the presence of common peaks at m/e 204, 203, 201, 189, 187, 175, 173, and 119; it was therefore thought possible that cyathin B^3 and cyathin C^3 could have the same carbon skeleton as in cyathin A^3.

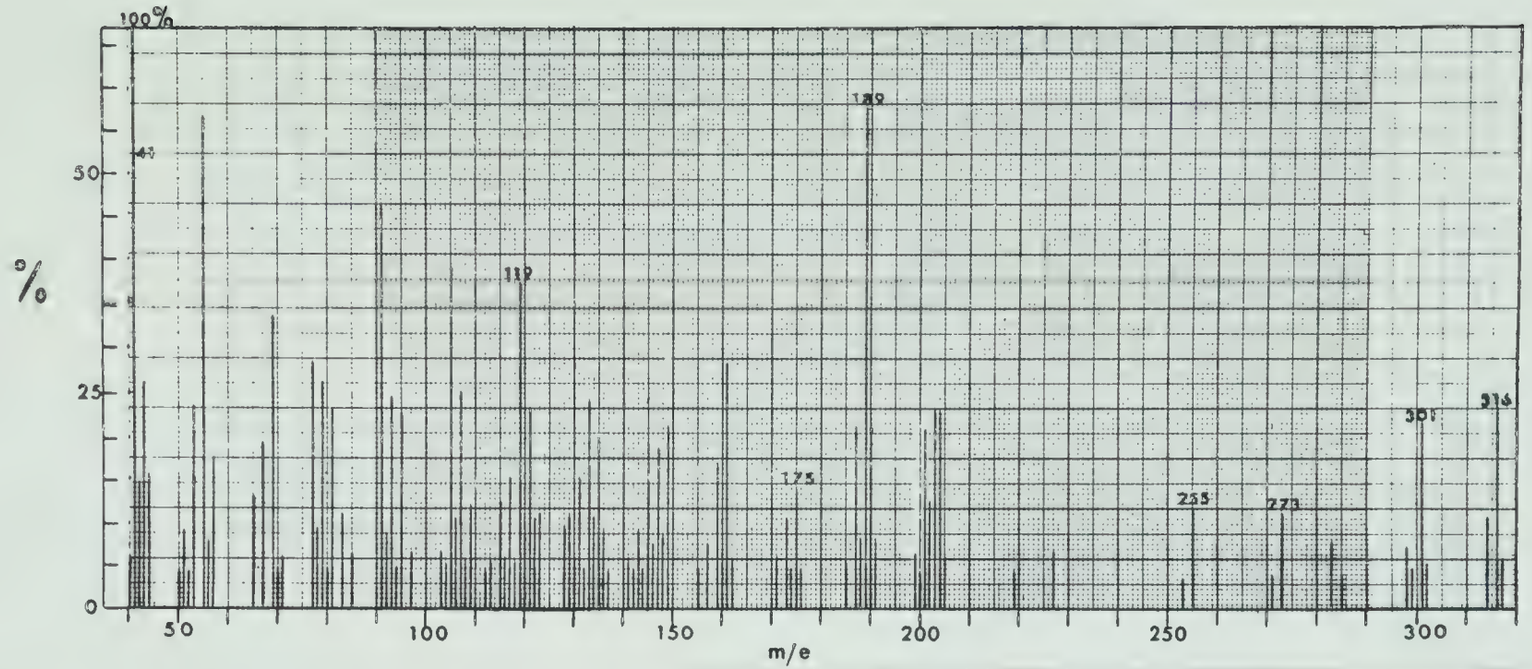
The infrared spectrum of cyathin B^3-C^3 in carbon tetrachloride solution (figure 2) shows both free and intermolecular hydrogen-bonded hydroxyl absorption at 3590 and 3360 cm^{-1}. The bands at 2810 and 2710 cm^{-1} are very characteristic of the C-H stretching vibration in aldehydes. Also the carbonyl band at 1693 cm^{-1} would suggest this aldehyde to be α,β-unsaturated. When the infrared spectrum of the material was determined in nujol mull (figure 3), a drastic difference in the carbonyl absorption is observed. The frequency of absorption has been lowered to 1657 cm^{-1} and the intensity is only about one-half of what it was in carbon tetrachloride solution. This can be rationalized by assuming there are <u>two</u> car-

TOP: FIGURE 1

Mass spectrum of cyathin B^3-C^3

BOTTOM: FIGURE 2

Infrared spectrum (CCl_4) of cyathin B^3-C^3


100%
50
25
0
%
47
119
139
175
235
273
301
316
50
100
150
200
250
300
m/e

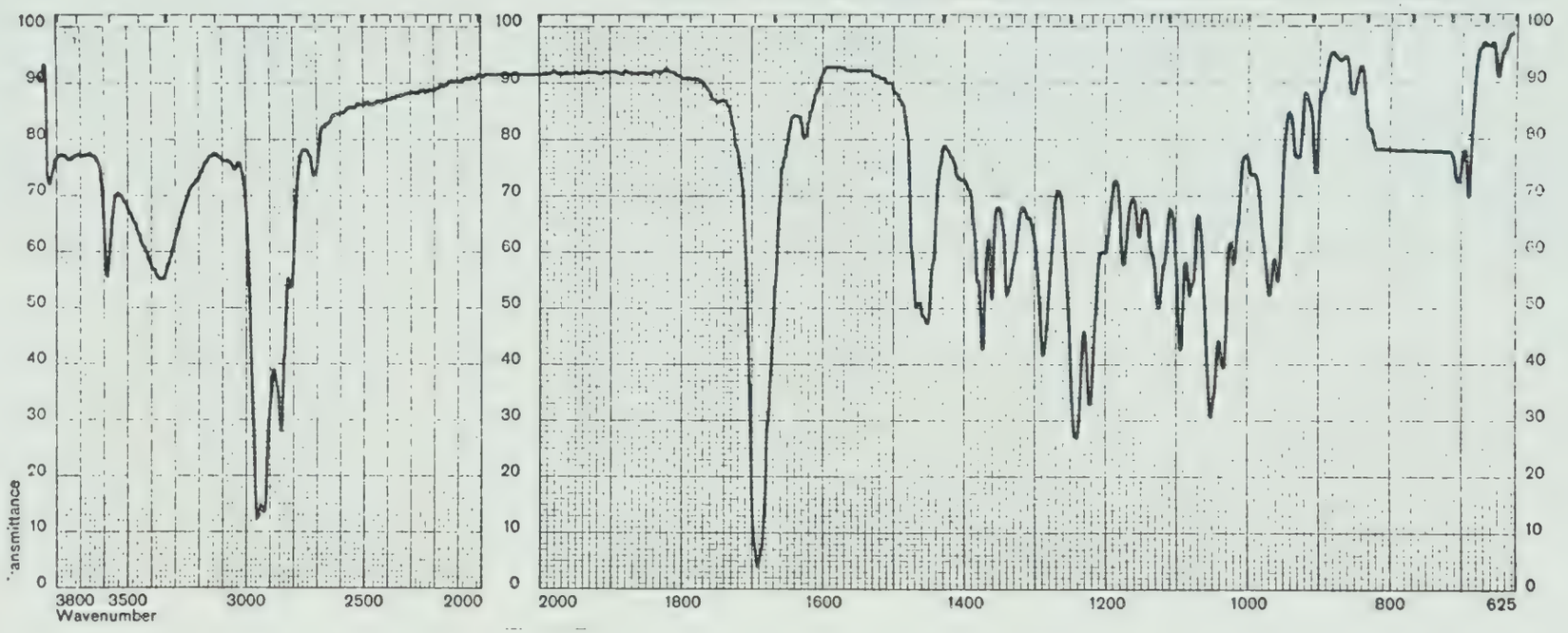
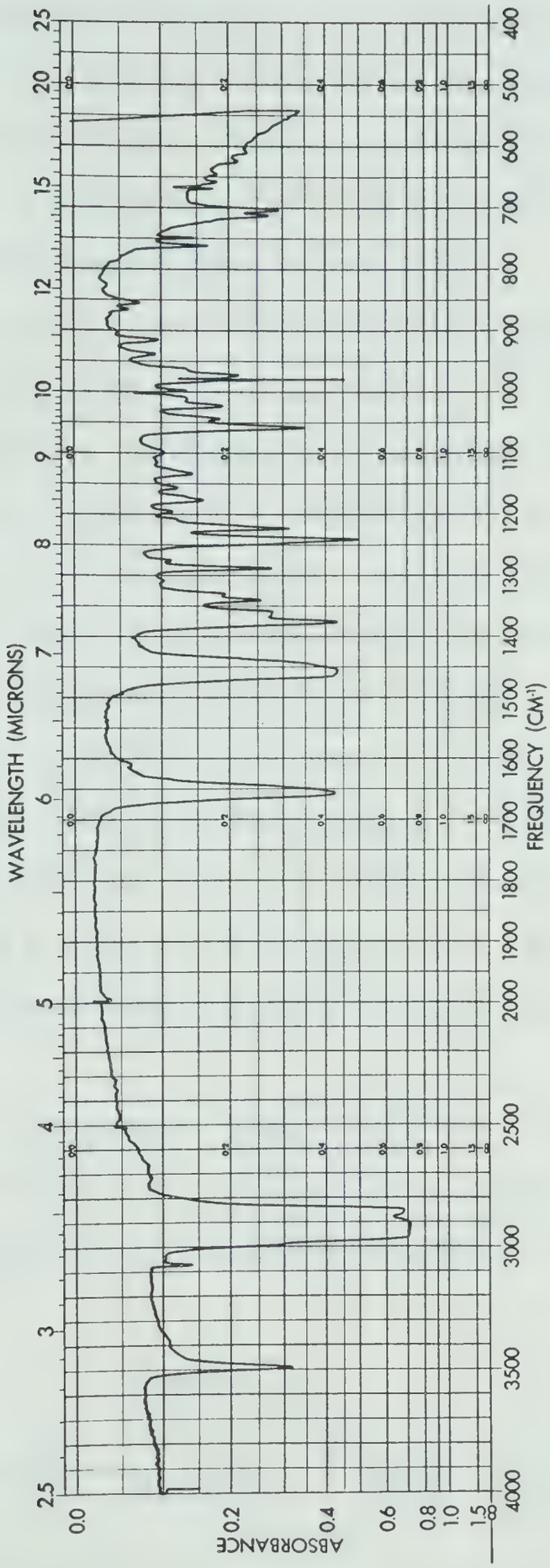

100
90
80
70
60
50
40
30
20
10
0
Transmittance
3800 3500
Wavenumber
3000
2500
2000
1800
1600
1400
1200
1000
800
625

FIGURE 3: Infrared spectrum (nujol mull) of cyathin B^3-C^3.

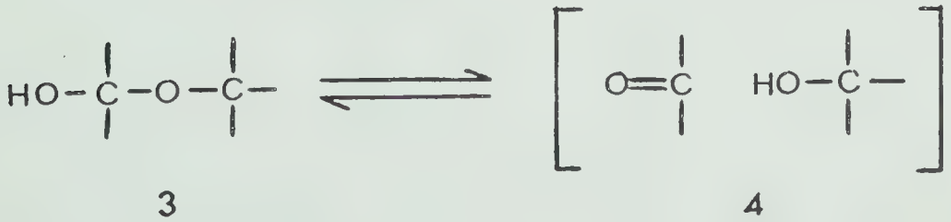

WAVELENGTH (MICRONS)
FREQUENCY (CM⁻¹)
ABSORBANCE
2.5
3
4
5
6
7
8
9
10
12
15
20
25
4000
3500
3000
2500
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
0.0
0.2
0.4
0.6
0.8
1.0
1.5
∞

bonyl oxygens absorbing in the solution spectrum, but that in the crystalline state one is "masked", possibly by hemiketal formation as in the case of cyathin A^3, so that there is only one carbonyl absorption. Also noteworthy are the bands at 3070 and 1610 cm^{-1}, indicating carbon-carbon double bond absorption.

The nuclear magnetic resonance (nmr) spectrum (figure 4) of the crystalline cyathin B^3-C^3 mixture determined in carbon tetrachloride solution proved to be quite complex. Especially confusing were the aldehydic signals at δ9.79, 9.56, and 9.42. If two compounds are present as indicated by mass spectrometry, then only two aldehyde peaks should be present in the nmr, unless each compound has two aldehydes and the four overlap in such a way as to give rise to three signals. This latter interpretation was ruled out when the spectrum was re-run in the presence of a drop of trifluoroacetic acid (figure 5). The aldehyde signals are now seen to converge to only one at δ9.75.

This phenomena, along with the ir spectra, may be explained in terms of an equilibrium between hemiketal (3) and hydroxyketone (4) forms as depicted in Scheme 1.

SCHEME 1



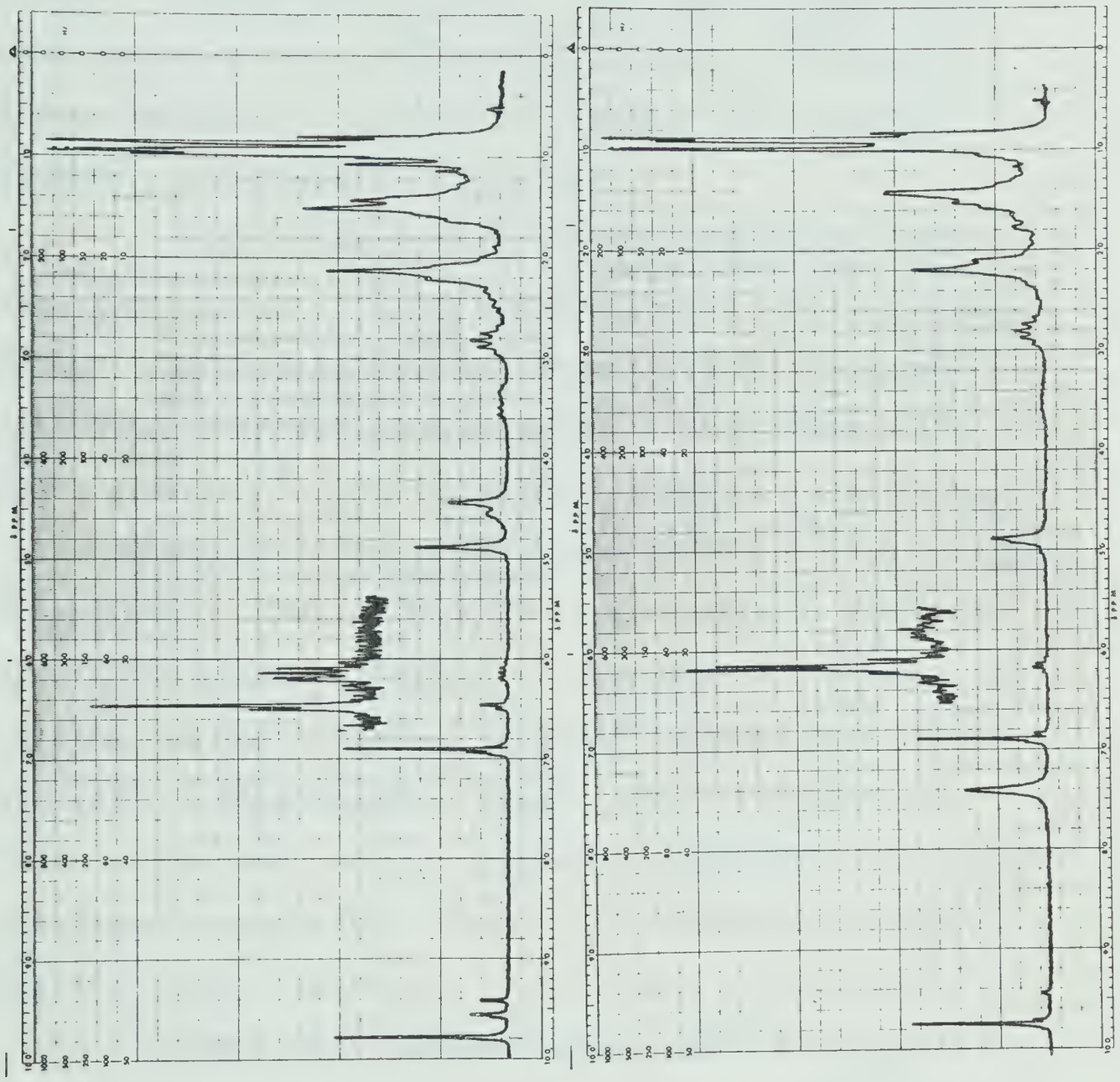
3 4

TOP: FIGURE 4

Nuclear magnetic resonance spectrum (CCl_4) of cyathin B^3-C^3

BOTTOM: FIGURE 5

Nuclear magnetic resonance spectrum (CCl_4 + trace CF_3COOH) of cyathin B^3-C^3



In the crystalline state, the two compounds in cyathin B^3-C^3 exist predominantly in the closed hemiketal form. In solution the two forms are present in more equal amounts. By the addition of acid, the equilibrium is shifted to the hemiketal form. Thus the nmr spectrum in carbon tetrachloride solution is actually the spectrum of four compounds: cyathin B^3, cyathin C^3, cyathin B^3 hemiketal, and cyathin C^3 hemiketal.

Also noteworthy in the nmr spectra is the eight-line signal at δ6.14 (without added acid), and the quartet signal at δ6.16 (with added acid). These are attributed to an isolated AB system in the dehydro compound, cyathin C^3. The eight-line signal is attributed to two overlapping AB quartets from cyathin C^3 and cyathin C^3 hemiketal. The addition of acid causes the hemiketal form to predominate, leading to only one AB quartet. The coupling constant observed (5.5 Hz) suggests the double bond is in a five-membered ring[9]. Also, the chemical shifts of the two protons suggest a double bond in a cyclopentadiene system rather than in a cyclopentene system (ref 10, p 138: δ5.60).

The signal at δ6.89 (with added acid) corresponds to the β proton of an α,β-unsaturated aldehyde (ref 10, p 139: δ6.90). This signal is seen as slightly broadened, possibly by a small coupling with the aldehyde proton or another proton.

The signal at δ4.90 (with added acid) is a

multiplet which corresponds to the C-11 proton in cyathin A^3 (ref 5, p 52: δ4.3). The difference of 0.6 ppm is attributed to "extra" unsaturation from the aldehyde carbonyl.

Absorption at δ2.80 (either spectra) gives rise to a septet (J = 7 Hz), characteristic of an isopropyl hydrogen. The region δ2.6 - 1.1 is very complex and not readily analyzable.

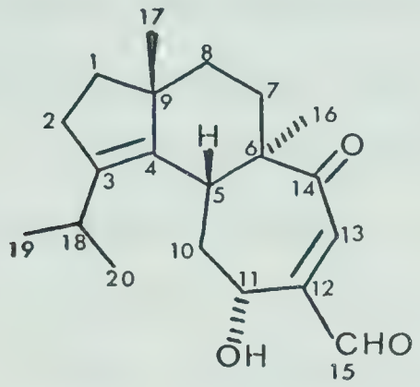
The methyl region in the spectrum determined without added acid is also very complex. When acid is added, the methyl region becomes somewhat better resolved, even though cyathin C^3 is still present. Centered at δ0.94 and 0.86 are two doublets coupled with the isopropyl hydrogen by 7 Hz. Two singlets at δ0.98 and 0.88 are also evident.

The nmr spectra also allows the estimation of the ratio of cyathin B^3 to cyathin C^3 in the crystalline material. This ratio is approximately 5 : 1.

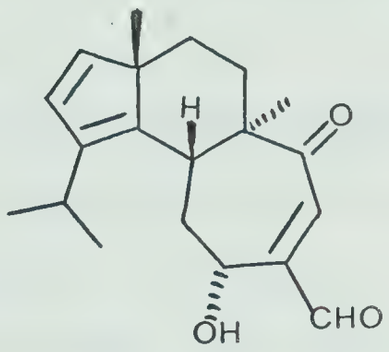
The uv spectrum (dioxane) of cyathin B^3-C^3 mixture showed a maxima at 233 nm (ϵ = 6800) and a shoulder at 350 nm (ϵ = 75). This also suggests the presence of an α,β-unsaturated ketone or aldehyde.

The evidence presented thus far suggests a close structural similarity between cyathin B^3-C^3 and cyathin A^3. Since cyathin B^3-C^3 contains an aldehyde function and cyathin A^3 contains a primary alcohol group not present in cyathin B^3-C^3, it seemed very possible that

the two series differed only in this way. Thus, structure 5 follows for cyathin B[3] and 6 for cyathin C[3].



5



6

Argentated silica gel thin-layer chromatography has been found useful in separating compounds differing only by the position of a double bond[11]. In cyathin A[3] - allocyathin B[3] chemistry, this was the method used for their separation[5]. The silver ions complex with the π-electrons of double bonds, and depending upon the number of double bonds and their freedom from steric hindrance, the compounds will be adsorbed to varing degrees. It was therefore hoped that the use of argentated tlc would greatly aid in the separation of cyathin B[3] from cyathin C[3].

When analytical tlc was carried out, indeed two spots did appear, thus helping to establish that actually two compounds were present in the crystalline material. When this type of chromatography was used on a preparative scale, two ill-defined bands were removed in three fractions as explained in the Experimental section. Ordinary tlc of these fractions showed other spots and streaks present at both higher and lower R_f. A preparative purification of the

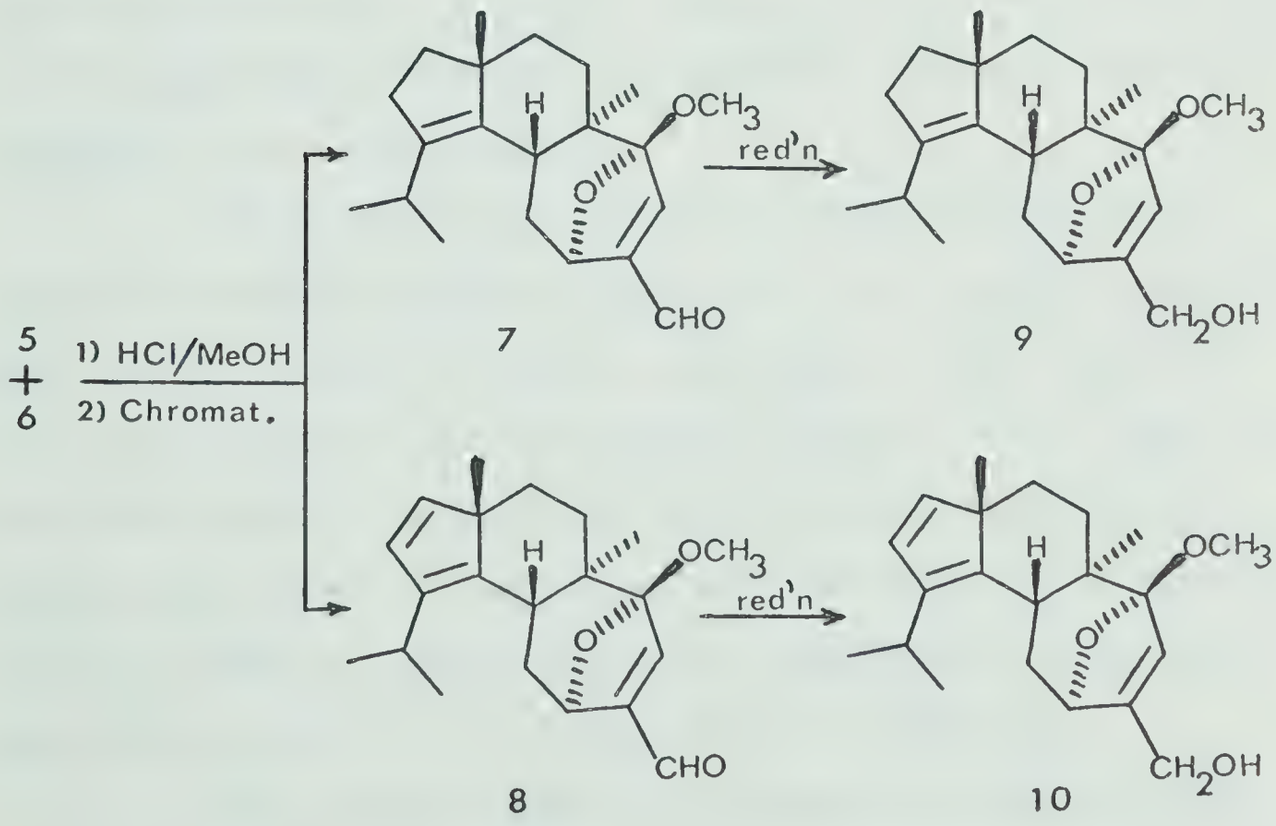
two cyathin B^3 rich fractions on ordinary plates gave crystalline material corresponding to partially purified cyathin B^3, but still contaminated by cyathin C^3. The loss of material during this process was extensive.

Since cyathin B^3-C^3 contains an aldehyde function, it is conceivable that the silver ions could have oxidized part of the material during the spotting of the plates, developement, drying, scraping off of the fractions, and elution of the material from the adsorbant -- all of which requires about four hours. Thus argentated tlc on the parent cyathin B^3-C^3 mixture was abandoned and another approach was sought to separate the two compounds.

2) Separation of Cyathin B^3-C^3 *via* their Methyl Ketals

At this point it became desirable to chemically prove the structures of cyathin B^3 and cyathin C^3. From previous work in the cyathin A^3 series, it was known that the hydroxyketone would form an internal methyl ketal when treated with methanol and anhydrous hydrogen chloride[5]. It was anticipated that cyathin B^3 and cyathin C^3 would behave similarly and that perhaps the two methyl ketals could be separated effectively without extensive loss of material. Reduction of the aldehyde function in each of these should then lead to the known cyathin A^3 and allo-cyathin B^3 methyl ketals, (9) and (10). Scheme 2 shows this sequence.

SCHEME 2



Indeed, when a sample of cyathin B^3-C^3 was treated with a saturated solution of anhydrous hydrogen chloride in methanol, analytical tlc showed only one spot at a higher R_f than the starting material. This material could not be induced to crystallize, but was chromatographically pure of contaminates and therefore used for spectral measurements as it was. All the spectral data (mass, ir, nmr, uv) agree with the methyl ketal structures for the mixture of cyathin B^3 and cyathin C^3, (7) and (8).

High resolution mass spectrometry gave $C_{21}H_{30}O_3$ for the apparent parent peak at m/e 330, which corresponds to the addition of the required unit CH_2 to cyathin B^3. The starting material used for this experiment was richer in cyathin C^3 than cyathin B^3, therefore the m/e 328 peak ($C_{21}H_{28}O_3$) has a greater intensity than the m/e 330 peak (see Experimental). A further discussion of the mass spectra of the separated cyathin B^3 and cyathin C^3 methyl ketals is presented in a later section.

The infrared spectrum in carbon tetrachloride solution shows no hydroxyl absorption, one carbonyl band at 1695 cm^{-1} of normal intensity, and bands at 2820 and 2720 cm^{-1} characteristic of the aldehyde moiety. Also, there are weak bands at 1625, 3060, and 3100 cm^{-1}, indicating double bond unsaturation; especially characteristic are the 3060 and 3100 cm^{-1} bands which are indicative of the cyclopentadiene moiety as in allocyathin B^3 chemistry[5].

The nuclear magnetic resonance spectrum of this

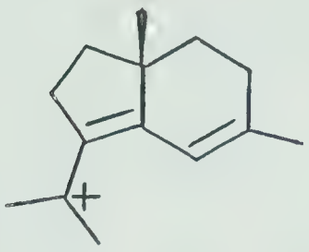
mixture of methyl ketals shows singlets at δ9.91 and 9.88, and also two methoxyl singlets at δ3.27 and 3.25 (ref 10, p 137: δ3.20 for methoxyl protons). The remaining data for the nmr spectrum is given in the Experimental.

At this point it was hoped that the two methyl ketals could be separated by chromatography. Ordinary tlc showed only one spot on silica gel (solvent system G). When an analytical plate coated with 10% silver nitrate on silica gel was developed, two spots were detected by viewing under uv light. The difference in R_f values of the two spots ($R_f(AgNO_3$, G) 0.62 and 0.55) lead to the anticipation that a preparative separation could be achieved, as long as decomposition did not occur as had happened with the parent cyathin B^3-C^3 material.

When the preparative work was carried out, the contact time of the material on the plates was kept to a minimum (about 1.5 hr). After development, two nicely separated bands were removed and immediately eluted from the adsorbant with ether. Analytical argentated tlc subsequently run on the two fractions indicated good separation and the absence of impurities. Numerous attempts were made to crystallize the two methyl ketals, but none were successful.

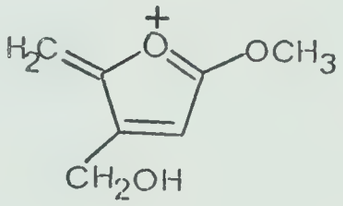
The mass spectrum of cyathin B^3 methyl ketal (7), $R_f(AgNO_3$, G) 0.62, is shown in figure 6. High resolution measurements on the parent peak (m/e 330) gave $C_{21}H_{30}O_3$ as the molecular formula. The base peak at m/e 189 has the composition $C_{14}H_{21}$ analogous to cyathin A^3 mass spectra

(ref 5, p 35). The most probable structure of this ion is 11.



11

The mass spectrum of cyathin A[3] methyl ketal is dominated by a peak at m/e 141, which has previously been assigned structure 12[5].



12

In cyathin B[3] methyl ketal, the electron withdrawing formyl group must drastically destabilize an ion of the type 12, since the peak at m/e 139 in the mass spectrum of cyathin B[3] methyl ketal has only an intensity of 6%. Instead, the charge remains with the hydrocarbon portion and gives rise to 11.

The infrared spectrum of cyathin B[3] methyl ketal (figure 7) shows absorption expected for the proposed structure: no hydroxyl absorption, aldehyde C-H stretch at 2810 and 2720 cm^{-1}, carbonyl absorption at 1693 cm^{-1}, and a sharp fingerprint region.

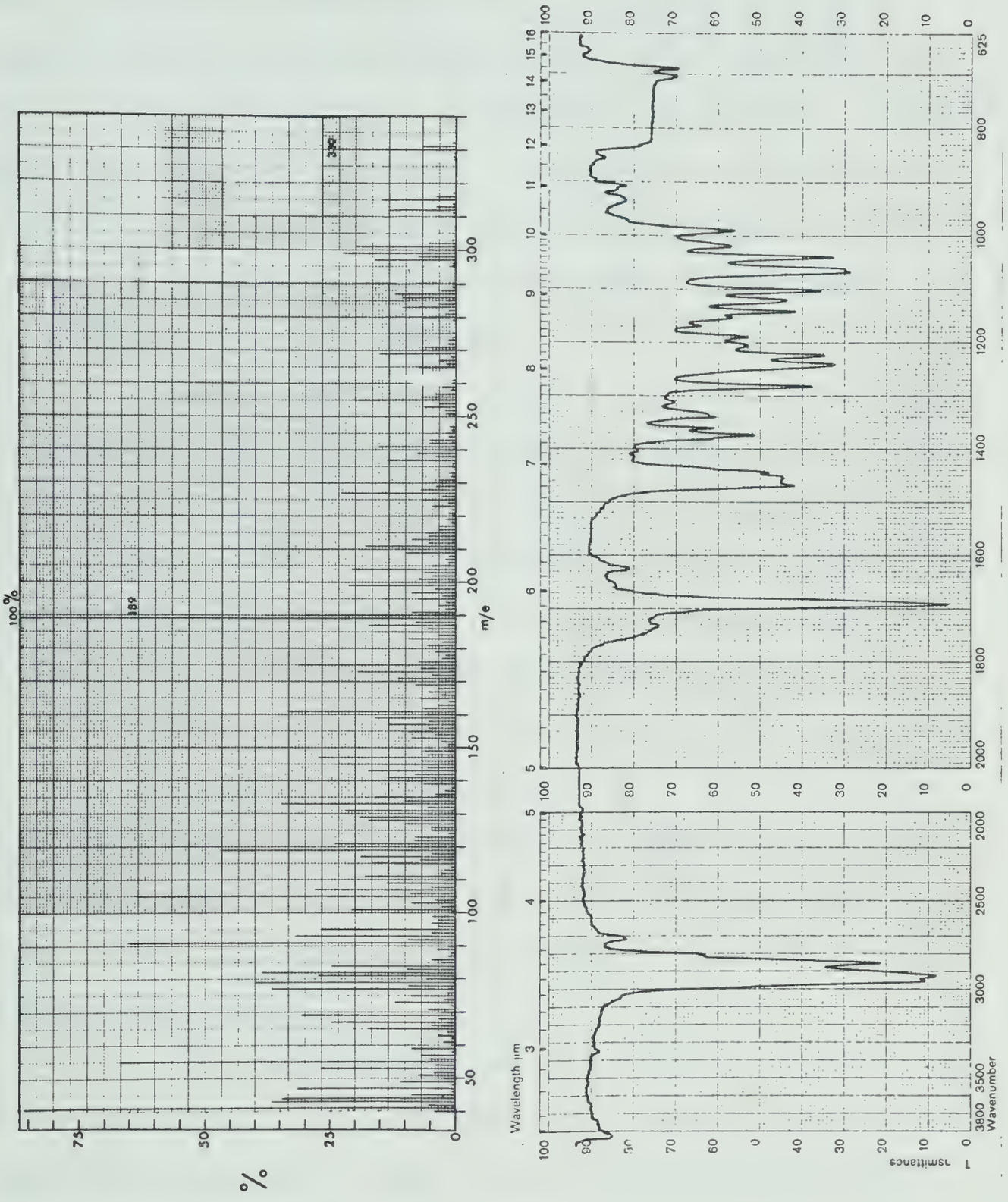
The nuclear magnetic resonance spectrum of this

TOP: FIGURE 6

Mass spectrum of cyathin B^3 methyl ketal

BOTTOM: FIGURE 7

Infrared spectrum (CCl_4) of cyathin B^3 methyl ketal


100 %
75
50
25
0
%
389
330
50
100
150
200
250
300
m/e
Wavelength µm
3
4
5
6
7
8
9
10
11
12
13
14
15
16
3800 3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625
Wavenumber
Transmittance

material determined in carbon tetrachloride solution (table I) also shows absorption consistent with the proposed structure. Especially characteristic is the singlet at δ3.27, representing the methoxyl absorption. Also the methyl region is more distinct than in previous nmr spectra, and four methyl groups can be analyzed. At δ0.90 and 0.94 are two singlets and at 0.88 and 0.99 are a pair of doublets with a coupling constant of 7 Hz each (coupled to the septet signal at δ2.90, isopropyl hydrogen).

The uv spectrum of cyathin B^3 methyl ketal shows the expected α,β-unsaturated carbonyl absorption at 233 nm (ϵ = 5300). The chromophore of the unsaturated system is also evident in the circular dichroism spectrum, which shows negative absorption peaks at 346 nm ($\Delta\epsilon$ = -0.97, $n\rightarrow\pi^*$ transition) and 235 nm ($\Delta\epsilon$ = -2.78, $\pi\rightarrow\pi^*$ transition).

The second component separated by ptlc, $R_f(AgNO_3$, G) 0.55, was cyathin C^3 methyl ketal (8). High resolution mass spectrometry gave the molecular formula $C_{21}H_{28}O_3$ for the parent peak at m/e 328. The mass spectrum (figure 8) shows a base peak at m/e 187, corresponding to the 1,2-dehydro analogue of 11.

The remaining spectra of cyathin C^3 methyl ketal are very similar to those of cyathin B^3 methyl ketal, except for those features due to the cyclopentadiene moiety present in cyathin C^3 methyl ketal.

The infrared spectrum (figure 9) shows weak bands at 3100 and 3050 cm^{-1}, along with an intense band at 700 cm^{-1}.

Table I. NMR Data for Cyathin B^3 Methyl Ketal and Cyathin C^3 Methyl Ketal

Signal	Shift of Cyathin B^3 Methyl Ketal in CCl_4 (δ)	Shift of Cyathin C^3 Methyl Ketal in CCl_4 (δ)	Multiplicity	Coupled with	Coupling constants
a	7.00	7.03	s	—	—
b	4.97	5.04	u	(g,j)	(?,3.5)
e	2.93	2.95	qq	s,t	7,7
f	2.3-2.1	2.22	dd	j,g	12,3
g	1.6-1.1	1.7-1.2	(ddd)	(j,f,b)	(13,3,?)
h,i	2.3-2.1	—	u	?	?
j	2.3-2.1	2.40	ddd	g,f,b	13,12,3.5
k,l	1.6-1.1	—	u	?	?
m,n,o,p	1.6-1.1	1.7-1.2	u	?	?
q	0.94	0.88	s	—	—
r	0.90	0.77	s	—	—
s	0.98	1.09	d	e	7
t	0.89	1.03	d	e	7
w	3.26	3.29	s	—	—
x	9.88	9.92	s	—	—
y	—	6.09	d	z	5.5
z	—	6.20	d	y	5.5

TOP: FIGURE 8

Mass spectrum of cyathin C^3 methyl ketal

BOTTOM: FIGURE 9

Infrared spectrum (CCl_4) of cyathin C^3 methyl ketal

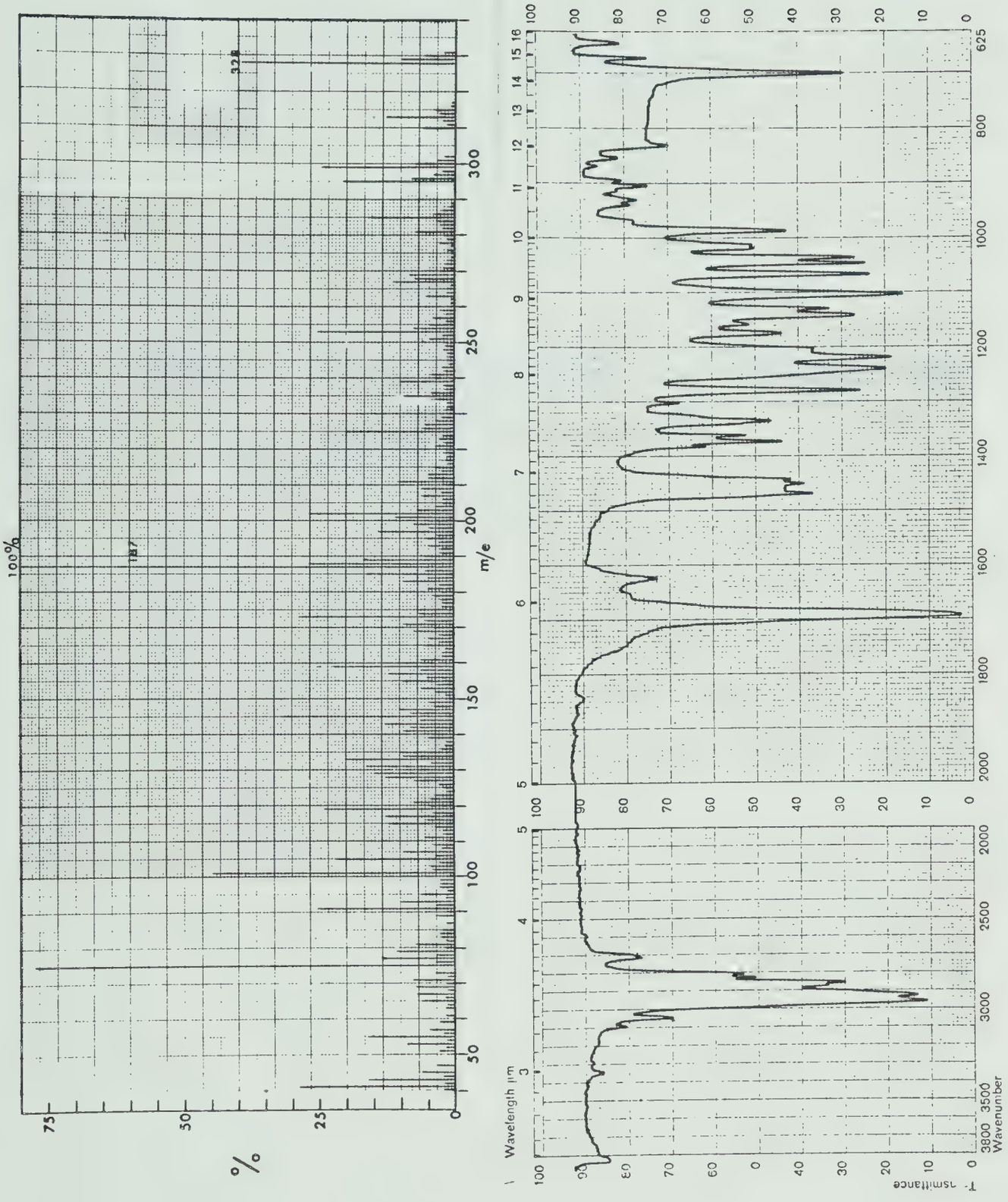
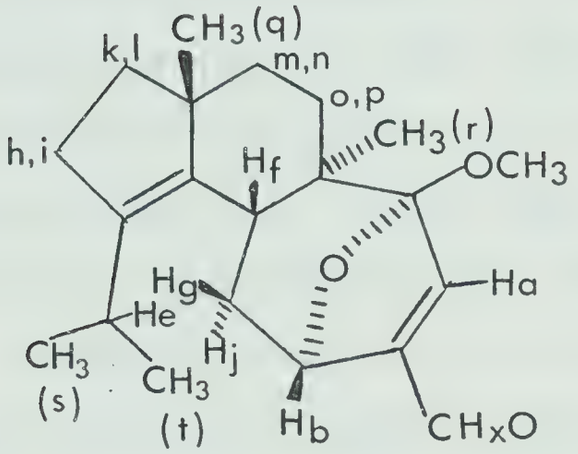

100%
187
328
75
50
25
0
%
50
100
150
200
250
300
m/e
Wavelength µm
3
4
5
6
7
8
9
10
11
12
13
14
15
16
100
90
80
70
60
50
40
30
20
10
0
T- Transmittance
3800
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625
Wavenumber

FIGURE 10: Proton designations for cyathin B^3 and cyathin C^3 methyl ketals



In cyathin C^3 series replace h,i with y and k,1 with z.

As mentioned before, these bands are regarded as characteristic of the cyclopentadiene moiety.

The nuclear magnetic resonance spectrum (table I) was analyzed in detail on one occasion since the spectrum showed sharp absorption lines and was devoid of impurities. Double resonance (decoupling) experiments were used to verify the coupling patterns.

Irradiation of the signal in the δ6.15 region left the remainder of the spectrum unchanged, thus verifying the isolated AB quartet nature of the y,z protons (see figure 10 for proton designations).

Irradiation of the narrow multiplet at δ5.04 (proton b) transformed the signal at δ2.19 (proton j) to a doublet of doublets (J = 12, 13 Hz), thus showing proton b and proton j to be coupled by 3.5 Hz. Also, the multiplet between δ1.2 - 1.6 was sharpened up, showing the presence of proton g by revealing its small coupling with proton b. The complexity of the δ1.2 - 1.6 region made further analysis very difficult in this region.

Irradiation of the signal due to the protons f and j (δ2.31) caused a drastic change in the pattern of the multiplet between δ1.2 - 1.6, but again the complexity did not allow these to be analyzed. On the other hand, when this region (δ1.53) was irradiated, the pattern for proton j was reduced to a doublet of doublets (J = 12, 3.5 Hz) showing the coupling of 13 Hz between the two geminal protons, g and j. Also, the signal for proton f was reduced to

a doublet (J = 12 Hz), verifying the coupling of 3 Hz between protons f and g.

When the septet at δ2.95 (proton e) was irradiated the two doublets at δ1.09 and 1.03 (J = 7 Hz) collapsed to two singlets, thus verifying the presence of the isopropyl group.

The uv spectrum of cyathin C^3 methyl ketal determined in iso-octane solution showed an absorption maxima at 236 nm (ϵ = 7400), analogous to that of cyathin B^3 methyl ketal, due to the α,β-unsaturated aldehyde. The only difference between the two spectra is the presence of a shoulder at 257 nm (ϵ = 4300) in the spectrum of cyathin C^3 methyl ketal, which is attributed to the cyclopentadiene chromophore. (Allocyathin B^3 methyl ketal shows λ_{max} 256 nm (ϵ = 4200) in iso-octane solution, ref 5, p 85.)

3) Correlation of Cyathin B^3 Methyl Ketal and Cyathin C^3 Methyl Ketal with Cyathin A^3 Methyl Ketal and Allocyathin B^3 Methyl Ketal

Reduction of α,β-unsaturated aldehydes with sodium borohydride is known to lead in most cases to the corresponding allylic alcohol (cinnamaldehyde → cinnamyl alcohol, ref 12, p 21). With a more active reducing agent such as lithium aluminum hydride, 1,4-addition often predominates to give the dihydro alcohol. Sodium borohydride seemed to be the first reagent of choice, although other complications could arise. Since the cyathin compounds including cyathin B^3-C^3 are known to be sensitive to basic conditions[13], the slightly alkaline sodium borohydride solution was considered with caution. It was anticipated however that reduction would be much faster than other nucleophilic reactions which are promoted by basic conditions.

When cyathin B^3 methyl ketal was treated with a solution of sodium borohydride in 95% ethanol, complete reduction was evident (tlc) after fifteen minutes. A spot at lower R_f was visualized by the H_2SO_4 charring method, but could not be detected by the uv method (in agreement with the loss of the chromophore). Also, the R_f value of this spot R_f(G) 0.31 is in good agreement with that reported for cyathin A^3 methyl ketal, R_f(G) 0.29. The sample was purified by preparative tlc. The following spectral data confirm that the product is cyathin A^3 methyl ketal (9).

High resolution mass spectrometry gave the molecular formula $C_{21}H_{32}O_3$ for the apparent parent peak at m/e

332. The mass spectrum (figure 11) of this material is dominated by the peak at m/e 141, which has been previously mentioned and assigned to the ion 12 in the cyathin A^3 methyl ketal mass spectrum. The mass spectrum of this material on the whole corresponds very well with the published spectrum of cyathin A^3 methyl ketal[5].

The infrared spectra of both the reduced material and authentic cyathin A^3 methyl ketal are given in figure 12. As anticipated, there has been loss of the carbonyl absorption and a gain of hydroxyl absorption at 3600 and 3490 cm^{-1} (weak). The fingerprint regions of the two compounds are in excellent correspondance with each other.

The nuclear magnetic resonance spectrum of the reduced material is also in very good agreement with that of the published spectrum of cyathin A^3 methyl ketal (see table II for the comparison). The two methylene protons of the primary hydroxyl are designated as protons c and d.

As expected, the uv spectrum of the reduced material shows only end absorption, since the reduction has removed the α,β-unsaturated aldehyde chromophore.

The absolute configuration of cyathin A^3 methyl ketal is known from X-ray diffraction studies [5,7]. The specific rotation of the reduced material is in excellent agreement with that for cyathin A^3 methyl ketal (-158° vs -154°), which confirms that they have the same absolute configuration.

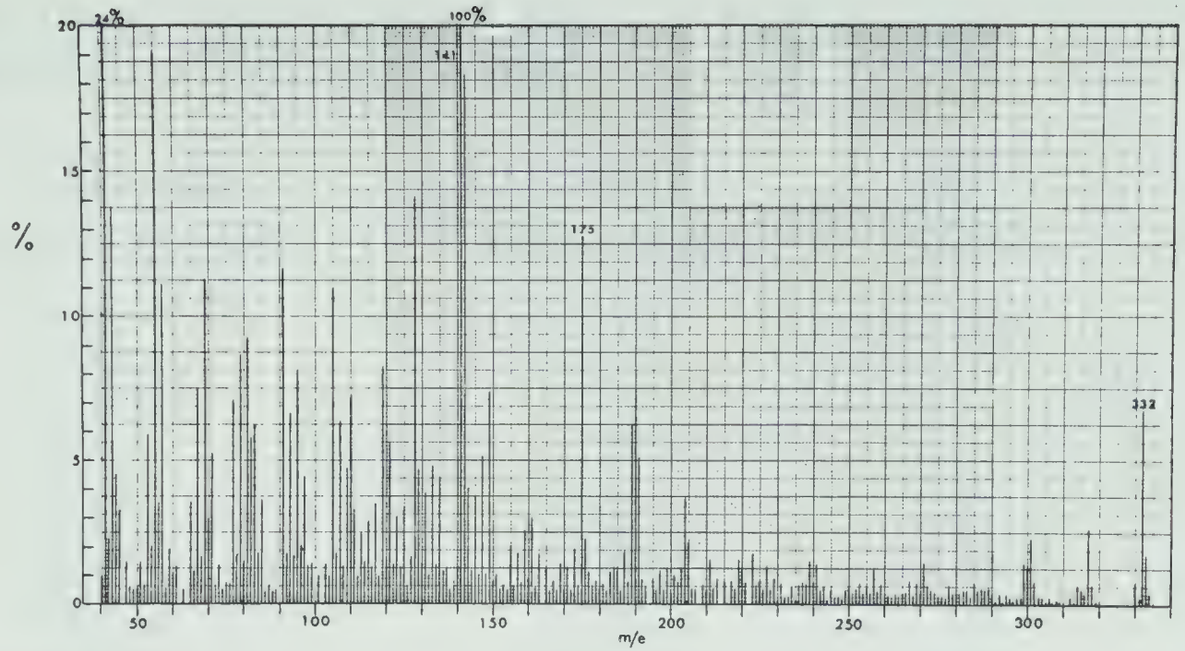
When cyathin C^3 methyl ketal was reduced with

TOP: FIGURE 11

Mass spectrum of reduced cyathin B^3 methyl ketal

BOTTOM: FIGURE 12

Infrared spectrum (CCl_4) of reduced cyathin B^3 methyl ketal (upper) and compared to published cyathin A^3 methyl ketal infrared spectrum (lower)


24%
100%
141
173
332
%
m/e

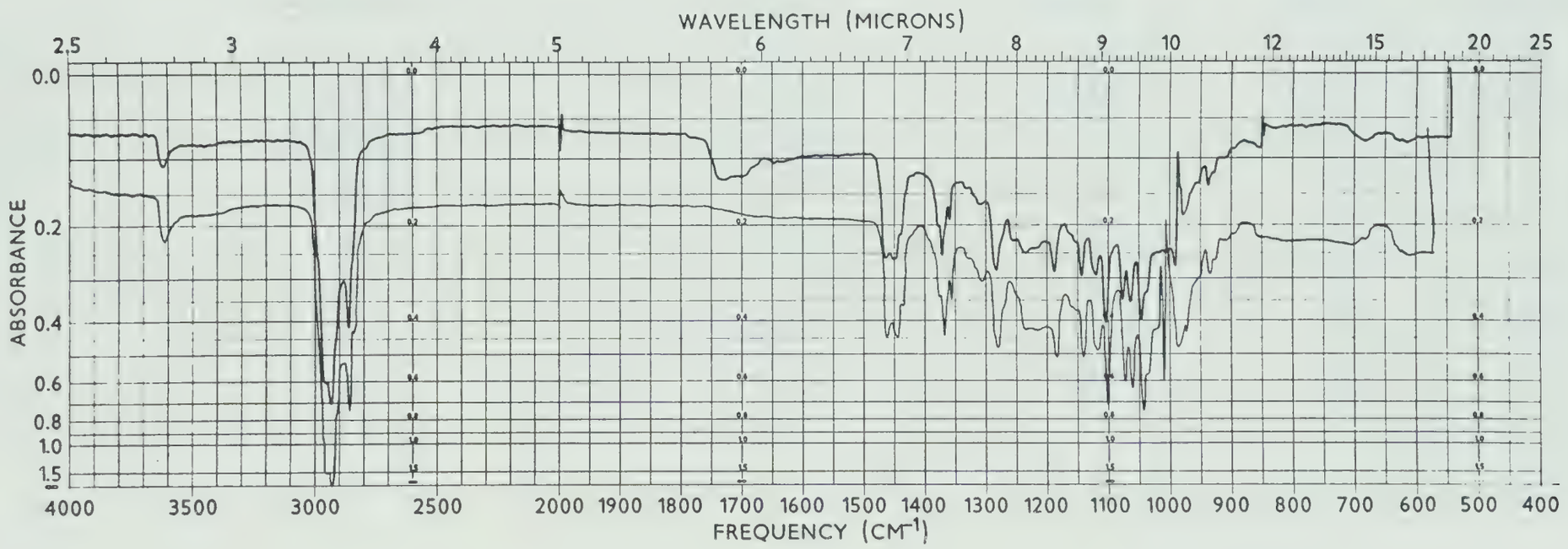

WAVELENGTH (MICRONS)
ABSORBANCE
FREQUENCY (CM⁻¹)

Table II. NMR Data for Cyathin A[3] Methyl Ketal obtained by Reduction of Cyathin B[3] Methyl Ketal and from a Published Source[5]

Signal	Shift of Reduction Product in C_6D_6 (δ)	Published Data (C_6D_6, δ)	Multiplicity	Coupled with	Coupling constants
a	5.83	5.86	dd	c,d	1.5,1.5
b	4.58	4.60	u	(j,?)	(3,?)
c	3.66	3.76	u	(d,a)	(16,1.5)
d	3.66	3.76	u	(c,a)	(16,1.5)
e	2.95	2.96	qq	s,t	6.5,6.5
f	2.4-2.1	2.44	(m)	(j,g,?)	(12,?)
g	1.8-1.2	1.35	(dd)	(j,f)	(12,?)
h,i	2.4-2.1	2.20	?	?	?
j	2.4-2.1	2.19	ddd	g,f,b	12,12,3
k,l,m,n,o,p	1.8-1.2	1.9-1.2	?	?	?
q	1.34	1.31	s	—	—
r	1.01	1.01	s	—	—
s	1.04	1.04	d	e	6.5
t	0.92	0.93	d	e	6.5
w	3.34	3.34	s	—	—

sodium borohydride, a spot at lower R_f than starting material was noted within fifteen minutes. This material could be visualized both by the H_2SO_4 charring and uv methods, and appeared as one spot on tlc, R_f(G) 0.38 (R_f(G) 0.39 reported for allocyathin B^3 methyl ketal). The following spectral data confirm that this material is identical with allocyathin B^3 methyl ketal (10).

High resolution mass spectrometry indicated the molecular formula $C_{21}H_{30}O_3$ for the parent peak at m/e 330. The mass spectrum of this material is shown in figure 13. All the major peaks are in good agreement with those in the mass spectrum of allocyathin B^3 methyl ketal (ref 5, p 84). Here again the peak at m/e 141 dominates the spectra.

The infrared spectra of both authentic allocyathin B^3 methyl ketal and the material obtained by reduction of cyathin C^3 methyl ketal are given in figure 14. The spectra are very similar to that of cyathin A^3 methyl ketal, except for the bands at 3100, 3050, and 700 cm^{-1}, attributable to the cyclopentadiene moiety.

The nuclear magnetic resonance spectra of known allocyathin B^3 methyl ketal and the material obtained by reduction are compared in table III, and are in good agreement with each other.

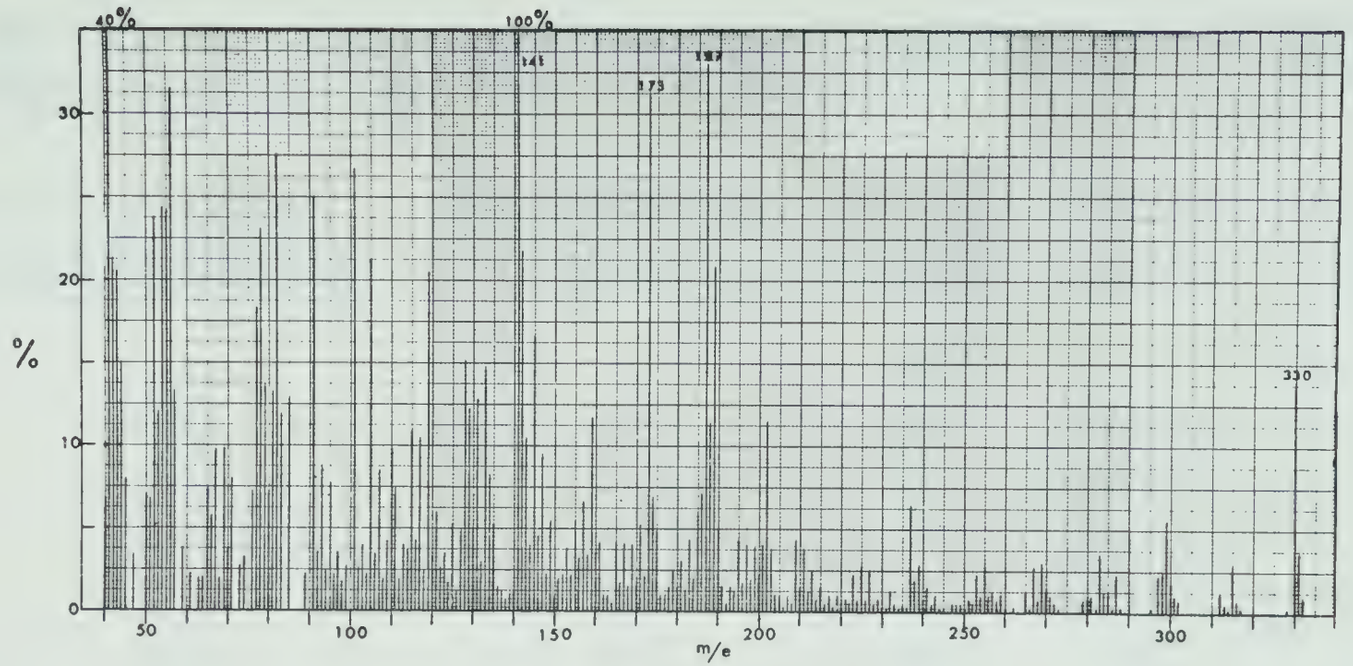
The uv spectrum of the reduced material in iso-octane solution shows a maxima at 255 nm (ϵ = 3500) due to the cyclopentadiene chromophore (reported spectrum of allocyathin B^3 methyl ketal: 256 nm (ϵ = 4200)). Also the cir-

TOP: FIGURE 13

Mass spectrum of reduced cyathin C^3 methyl ketal

BOTTOM: FIGURE 14

Infrared spectrum (CCl_4) of reduced cyathin C^3 methyl ketal (upper) and compared to published allocyathin B^3 methyl ketal infrared spectrum (lower)


40%
100%
145
173
187
330
%
30
20
10
0
50
100
150
200
250
300
m/e

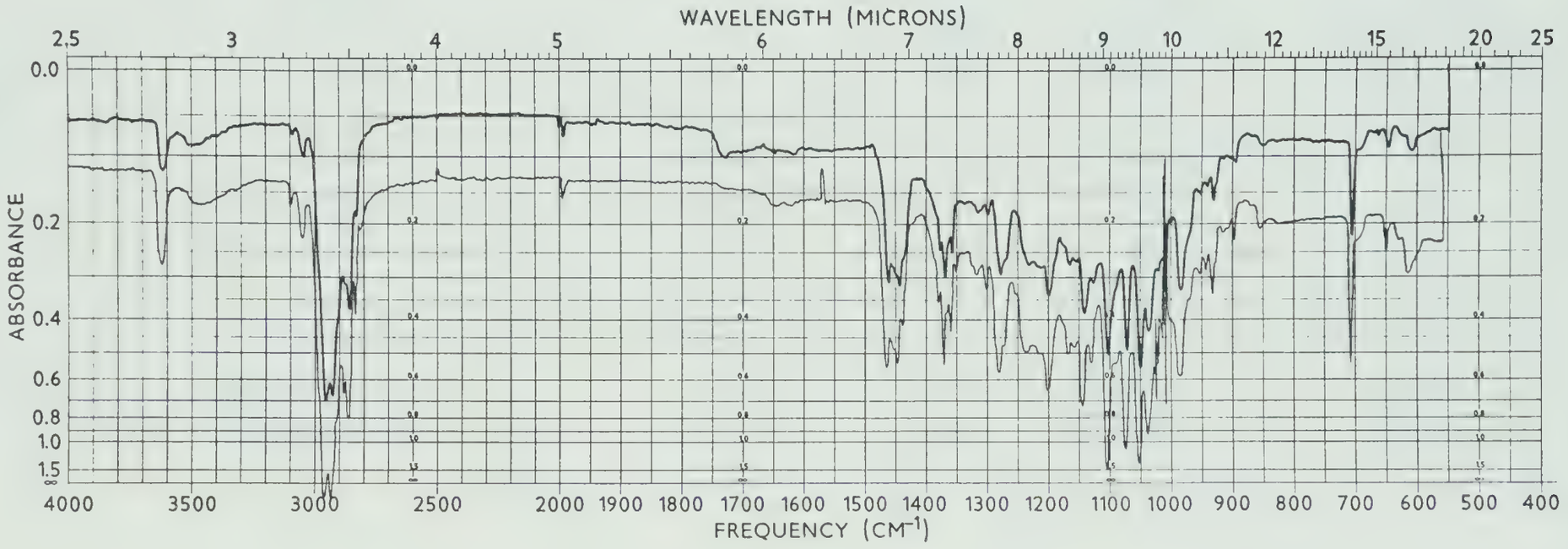

WAVELENGTH (MICRONS)
2.5
3
4
5
6
7
8
9
10
12
15
20
25
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
1.0
1.5
4000
3500
3000
2500
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM⁻¹)

Table III. NMR Data for Allocyathin B[3] Methyl Ketal obtained by Reduction of Cyathin C[3] Methyl Ketal and from a Published Source[5]

Signal	Shift of Reduction Product in C_6D_6 (δ)	Published Data (C_6D_6, δ)	Multiplicity	Coupled with	Coupling constants
a	5.88	—	dd	c,d	1.5,1.5
b	4.61	4.72	u	(j)	(3)
c	3.73	3.86	u	(d,a)	(14,1.5)
d	3.73	3.86	u	(c,a)	(14,1.5)
e	3.00	3.05	qq	s,t	7,7
f	2.62	2.68	dd	j,g	12.5,3.5
g	1.8-1.1	?	(dd)	(j,f)	(12.5,3.5)
j	2.33	2.48	ddd	f,g,b	12.5,12.5,3
m,n,o,p	1.8-1.1	1.8-1.1	m	?	?
q	1.18	1.18	s	—	—
r	1.00	1.02	s	—	—
s	1.14	1.16	d	e	7
t	1.10	1.12	d	e	7
w	3.35	3.41	s	—	—
y	blanked out	—	(d)	(z)	(5.5)
z	blanked out	—	(d)	(y)	(5.5)

cular dichroism spectra are in good agreement: reduced material vs known, $\Delta\epsilon_{253}$ -3.46 vs $\Delta\epsilon_{252}$ -3.6, respectively.

The specific rotation values at 589 nm are in only fair agreement (reduced material vs known, -179° vs -230°, respectively). This lack of agreement may be partially explained by the fact that the small quantities used, number of transfers, cell changes, etc., give rise to several opportunities for errors. This error has been estimated to be up to 50% when quantities of 2 mg are used. The agreement of the sign of rotation and the cd spectrum insures the absolute configuration.

Thus the structures of cyathin B[3] and cyathin C[3] are proven to be 3 and 4, respectively.

Further Characterization of Cyathin B^3 and Cyathin C^3

1) Initial acetylation experiments

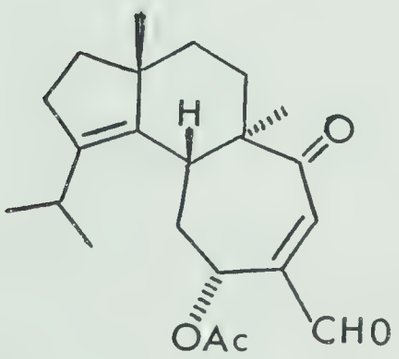
At one time it was anticipated that simple acetylation of the alcohol moieties of cyathin B^3-C^3 mixture would aid in their separation and further characterization. When the acetylation was actually conducted (see Experimental), four new spots of higher R_f than starting material were noted on analytical tlc. Acetylation was anticipated to give only two spots: the acetylated derivatives of cyathin B^3 and cyathin C^3. A preparative separation yielded four overlapping bands which were removed in the usual manner.

The least polar compound showed no hydroxyl or acetate carbonyl absorption in the infrared. Mass spectrometry showed an apparent parent peak at m/e 298. The only logical assumption to make was that the acetylation procedure had led to dehydration; this material will be discussed in detail in the next section.

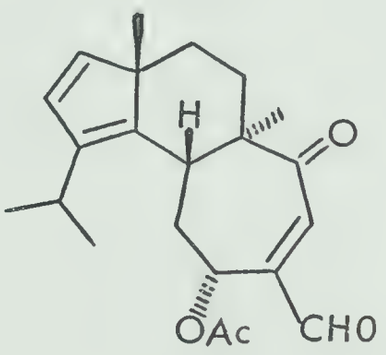
The two bands of intermediate polarity were shown to consist mainly of a material consistent in properties with O-acetylcyathin B^3 (13). Only infrared and mass spectrometry were used to characterize this compound. The infrared spectrum showed no hydroxyl absorption, but a band at 1745 cm^{-1} (acetate), and the usual aldehyde absorption bands at 2810, 2710, and 1710 cm^{-1} were present. The band at 1680 cm^{-1} was attributed to the ketone carbonyl in 13,

since an α,β-unsaturated ketone would be expected to be of lower frequency than an α,β-unsaturated aldehyde (ref. 10, pp. 87-88). The mass spectrum of this material showed a parent peak at m/e 358, consistent with the addition of the unit C_2H_2O to cyathin B^3.

The band of highest polarity showed characteristics consistent with O-acetylcyathin C^3 (14). Again only infrared and mass spectrometry were used to characterize the material. The infrared spectrum showed peaks similar to those of O-acetylcyathin B^3, and the mass spectrum showed a parent peak at m/e 356, consistent with the addition of the unit C_2H_2O to cyathin C^3.



13



14

The reason that the four compounds visualized by analytical tlc were not all isolated is because of the difficult preparative separation due to the similar R_f values of several of the spots. It is

assumed that the fourth compound would be the anhydro analogue of cyathin C^3 and that this compound, probably in small amounts, was "lost" in the O-acetylcyathin B^3 band during the preparative separation.

2) Anhydrocyathin B^3 and Anhydrocyathin C^3

A subsequent acetylation experiment was undertaken to explore the unexpected dehydration of cyathin B^3-C^3. A 10° higher temperature was used, and the acetylation was allowed to proceed until the four initial products as judged by tlc, converged into only the two of highest R_f value (see Experimental for the procedure). Work-up and preparative tlc separation of the two components gave two fractions, each chromatographically pure.

The compound of lowest polarity corresponded to the material isolated as the top fraction in the previous acetylation experiment, and was tentatively assigned structure 15 for anhydrocyathin B^3. This material gave the molecular formula $C_{20}H_{26}O_2$ by high resolution mass spectrometry. Its mass spectrum (figure 15) also shows m/e 283 to be the base peak ($M^+ - CH_3$).

The infrared spectrum (figure 16) shows aldehydic absorption at 2800, 2700, and 1720 cm^{-1}, ketonic carbonyl absorption at 1693 cm^{-1}, and carbon-carbon double bond absorption at 1623 and strongly at 1583 cm^{-1}. The band near 1770 cm^{-1} is attributed to acetate impurity.

The nuclear magnetic resonance spectrum of the dehydrated material was very informative (Table IV). Only two olefinic protons are present,

TOP: FIGURE 15

Mass spectrum of anhydrocyathin B^3

BOTTOM: FIGURE 16

Infrared spectrum (CCl_4) of anhydrocyathin B^3

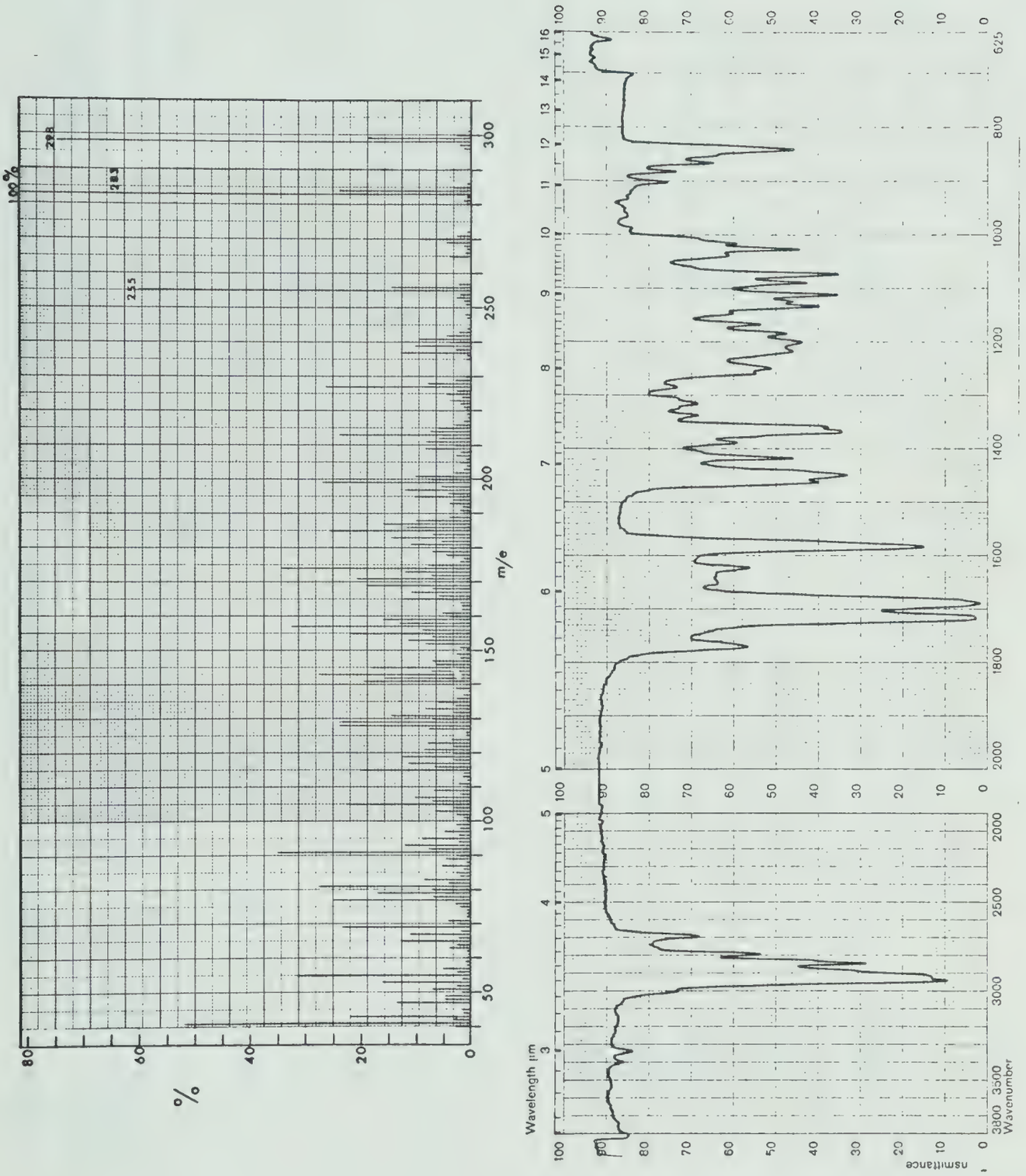
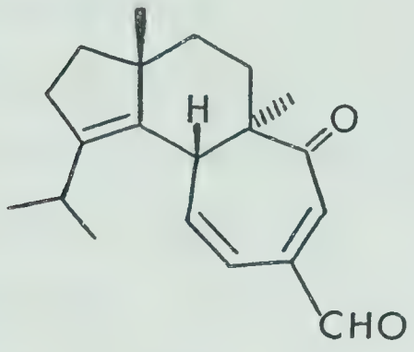

100%
298
283
255
%
m/e
Wavelength µm
Wavenumber
nsmittance

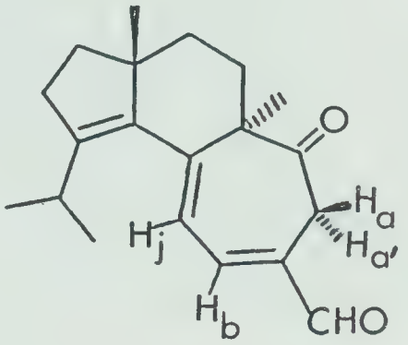
TABLE IV NMR DATA OF ANHYDROCYATHIN B[3] AND ANHYDROCYATHIN C[3]

Signal	Shift of anhydrocyathin B[3] in CCl_4 (δ)	Shift of anhydrocyathin C[3] in CCl_4 (δ)	Multiplicity	Coupled with	Coupling Constants(Hz)
a	3.09	3.04	dd	a',b	11, 1.5
a'	3.69	3.80	d	a	11
b	6.81	6.86	dd	j,a'	6, 1.5
e	2.81	2.85	qq	s,t	7, 7
h,i	2.4-1.1	----	u	?	?
j	5.98	6.00	d	b	6
k,l	2.4-1.1	----	u	?	?
m,n,o,p	2.4-1.1	2.4-1.2	u	?	?
q	1.06	0.97	s	----	----
r	0.95	0.94	s	----	----
s	0.99	1.11	d	e	7
t	0.93	1.00	d	e	7
x	9.42	9.43	s	----	----
y	----	6.36	d	z	5.5
z	----	6.29	d	y	5.5

and this immediately rules out structure 15. It was therefore speculated that double bond migration had occurred during the dehydration to give a more conjugated system, such as 16.



15



16

The two methylene protons (Ha, Ha') alpha to the ketonic carbonyl appear at δ3.09 and 3.69. One is a doublet coupled only to its geminal partner (J=11 Hz), while the other is a doublet of doublets (J=11,1.5 Hz) showing a longer range coupling along with the geminal coupling. When a model of structure 16 was constructed, it was seen that either Ha or Ha' could be in a "W" coupling path with proton b for allylic coupling, depending upon the orientation of the seven-membered ring. This being so makes the assignment of Ha and Ha' as beta and alpha to the plane of the molecule arbitrary. When the signal at δ6.81 (proton b) was irradiated and the spectrum rerun, the signal due to Ha was seen to collapse to a simple doublet (J=11 Hz), thus showing

the small allylic coupling between protons a and b.

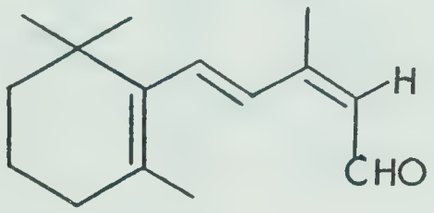
The two olefinic protons show absorption at δ6.81 and 5.98. The signal at δ6.81 appears as a doublet of doublets (J=6,1.5 Hz), and is assigned as proton b. The signal at δ5.98 appears as a doublet (J=6 Hz), and corresponds to proton j. The chemical shift of proton j is in good agreement with the gamma proton of an α,β,γ,δ-unsaturated carbonyl system (ref. 10, p.139; δ6.05). Irradiation of the signal at δ6.81 reduced the signal for proton j (δ5.98) to a singlet, verifying the olefinic coupling of 6 Hz.

The remainder of the spectrum, including the methyl region and the aldehydic proton are in good agreement with the structure 16 for anhydrocyathin B[3].

At this point the infrared spectrum (figure 16) should be re-evaluated. An absorption band at 1420 cm^{-1} is apparent, and this is very characteristic of the scissoring vibration of a methylene group alpha to a carbonyl (ref. 10, p. 107), which is present in structure 16. Also, the carbonyl bands should have the opposite designations from those given previously. The unconjugated ketone would be expected to absorb near 1720 cm^{-1}, while the heavily conjugated aldehyde would be expected to be at the lower frequency, 1693 cm^{-1}.

The uv spectrum in iso-octane solution showed two absorption maxima: 325 nm (ε=12,100) and 259 nm (ε=6,900). Although not a perfect model,

cis-β-ionylideneacetaldehyde 17 shows a similar spectrum[14] (cyclohexane solution): 309 nm (ε=11,700) and 264 nm (ε=10,300).

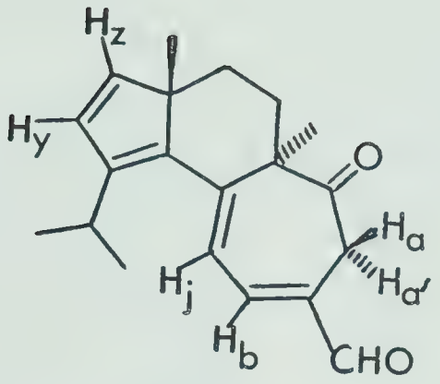


17

Since anhydrocyathin B[3] has one more substituent on the conjugated system than does the model compound (five as compared to four), the addition of ~18 units according to Woodward's rules[15] would give 327 nm for the maxima of the model compound; close agreement with the observed 325 nm.

The other compound isolated from the acetylation-dehydration experiment showed slightly greater polarity on tlc. This compound crystallized after being allowed to stand in the fridge for approximately one week. Recrystallization was effected with ether-pentane to give light yellow needles, m.p. 105.5 - 107°. All of the spectral data suggest that this compound is anhydrocyathin C[3] (18).

High resolution mass spectrometry gave $C_{20}H_{24}O_2$ for the parent peak at m/e 296, which also is the base peak (figure 17).



18

The infrared spectrum (figure 18) is very similar to that of anhydrocyathin B[3]. The extended conjugation has lowered the frequency of the olefinic C-H stretch bands due to the cyclopentadiene moiety so that they fall in the aliphatic C-H stretch region although the characteristic 700 cm^{-1} band is still present.

The nuclear magnetic resonance spectrum (Table IV) was a close replica of that of anhydrocyathin B[3], except for those portions due to the cyclopentadiene moiety. Thus the y,z protons show as a broadened singlet at δ6.33 and the complex region from δ2.2-1.2 integrates to four less protons.

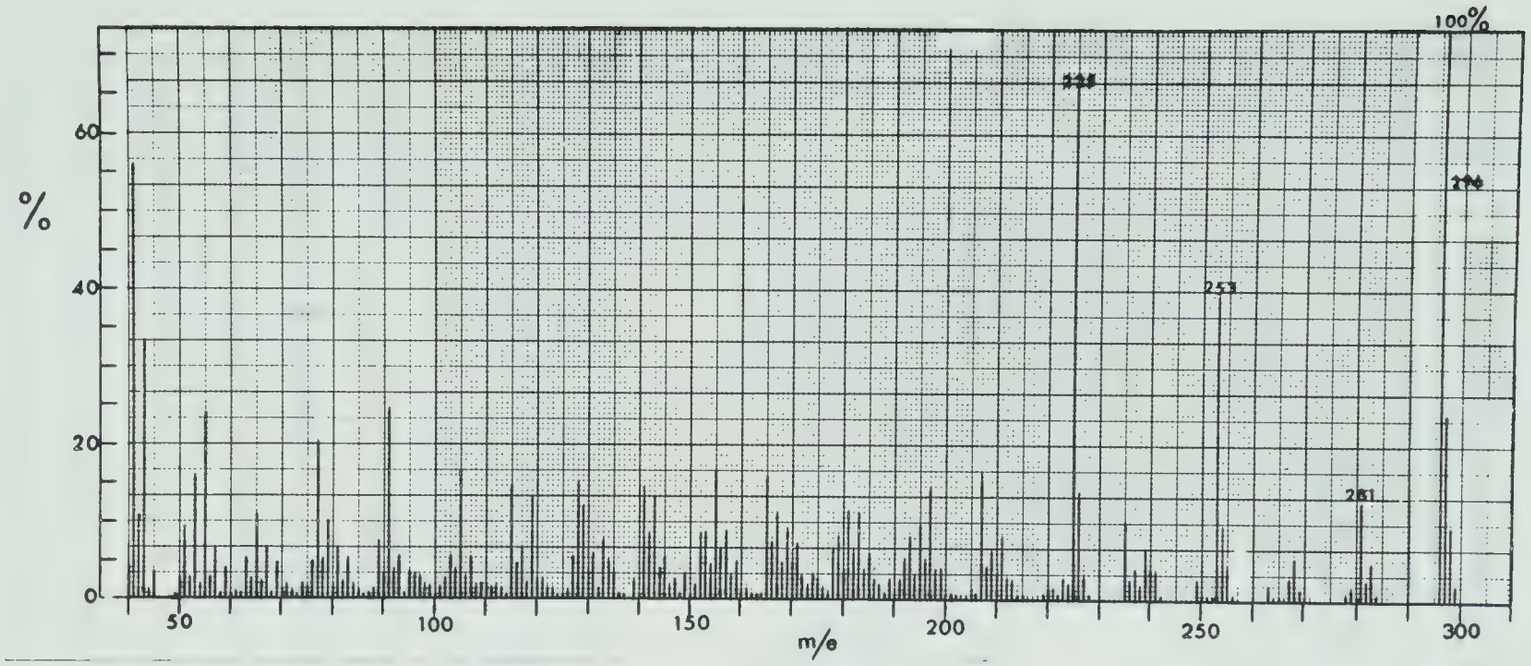
The uv spectrum showed a more extended chromophore than in anhydrocyathin B[3], absorbing at 345 nm (ε=10,400) and 264 nm (ε=6,000), accounting for the yellow color of the compound.

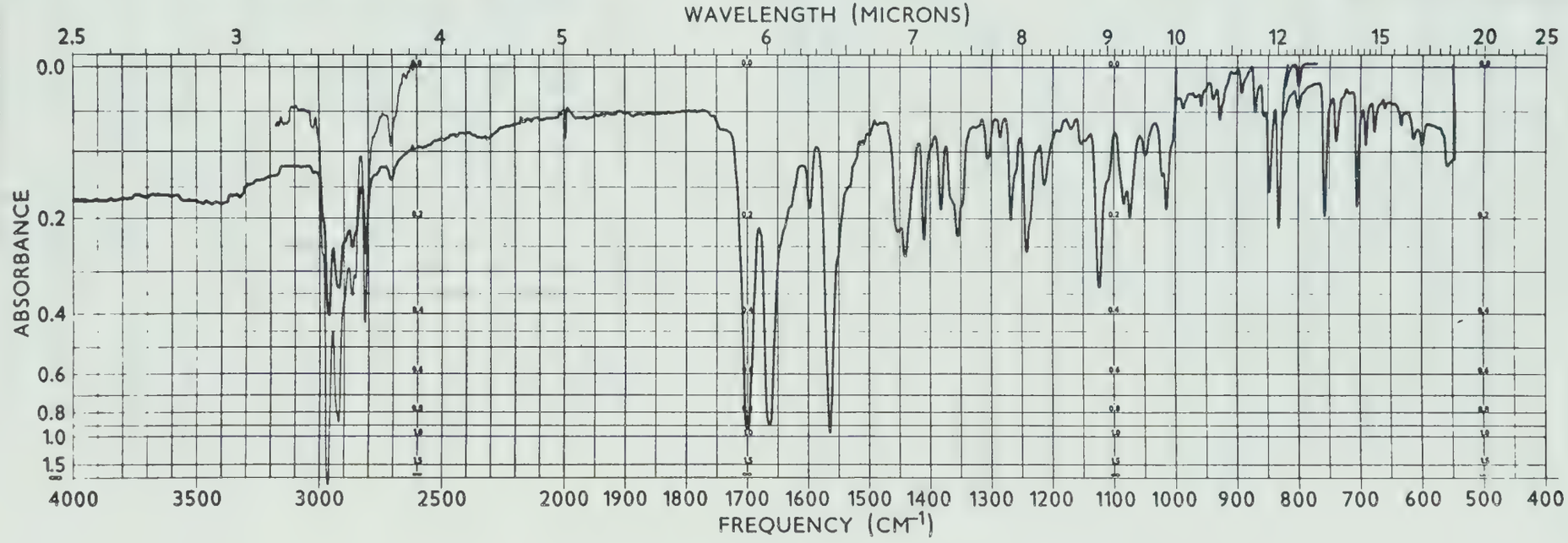
TOP: FIGURE 17

Mass spectrum of anhydrocyathin C^3

BOTTOM: FIGURE 18

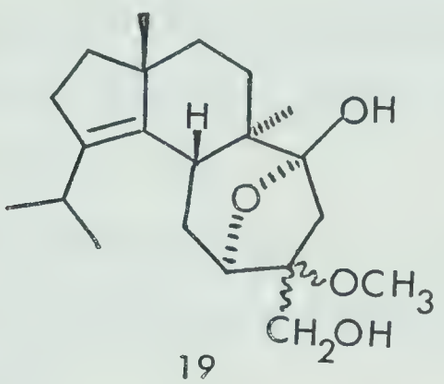
Infrared spectrum (nujol mull) of anhydrocyathin C^3


100%
%
60
40
20
0
253
294
261
50
100
150
200
250
300
m/e


WAVELENGTH (MICRONS)
2.5
3
4
5
6
7
8
9
10
12
15
20
25
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
1.0
1.5
4000
3500
3000
2500
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
FREQUENCY (CM-1)

3) Methanol Adduct of Cyathin B[3]

It was known previously that the cyathin compounds were sensitive to alkaline conditions. A methanol addition promoted by base catalysis has been reported for cyathin A[3] in which the α,β-unsaturated ketone moiety is attacked in Michael fashion to give 19 (both epimers)[13]. It was therefore interesting to



19

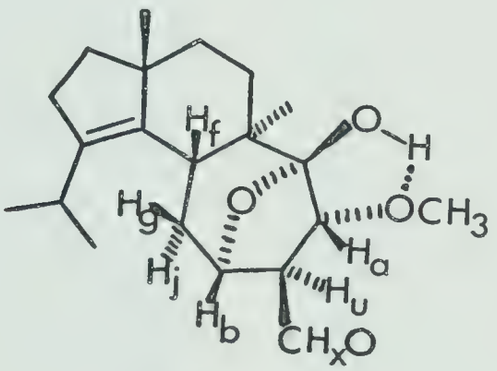
see whether and how the addition would occur to the cross-conjugated keto-aldehyde system of cyathin B[3].

After some experimenting, a method was found which led to a clean reaction of cyathin B[3]-C[3] in alkaline methanol solution. Approximately 0.1% potassium carbonate in methanol was sufficient to give complete conversion to a compound whose R_f value on tlc was slightly higher than cyathin B[3]-C[3], and which could not be detected by the uv method. All of these reactions were carried out under an atmosphere of nitrogen, since in the presence of air a very impure product was obtained as revealed by tlc (probably due

to peroxide formations by the addition of oxygen). The cyathin C^3 adduct in the reaction mixture was then converted to cyathin B^3 adduct by selective hydrogenation of the 1,2-double bond.

Also noteworthy is the fast rate of the methanol addition reaction. Within ten minutes the reaction had gone to completion as judged by tlc, whereas in the case of cyathin A^3, seventeen hours were needed, under much more basic conditions (2% KOH/methanol). Epimeric mixtures were not obtained in the case of cyathin B^3-C^3 as judged by tlc.

The spectral data to be subsequently described support structure 20 for the addition product of methanol to cyathin B^3.



20

High resolution mass spectrometry gave the expected molecular formula $C_{21}H_{32}O_4$ for the parent peak at m/e 348. The mass spectrum is reproduced in figure 19.

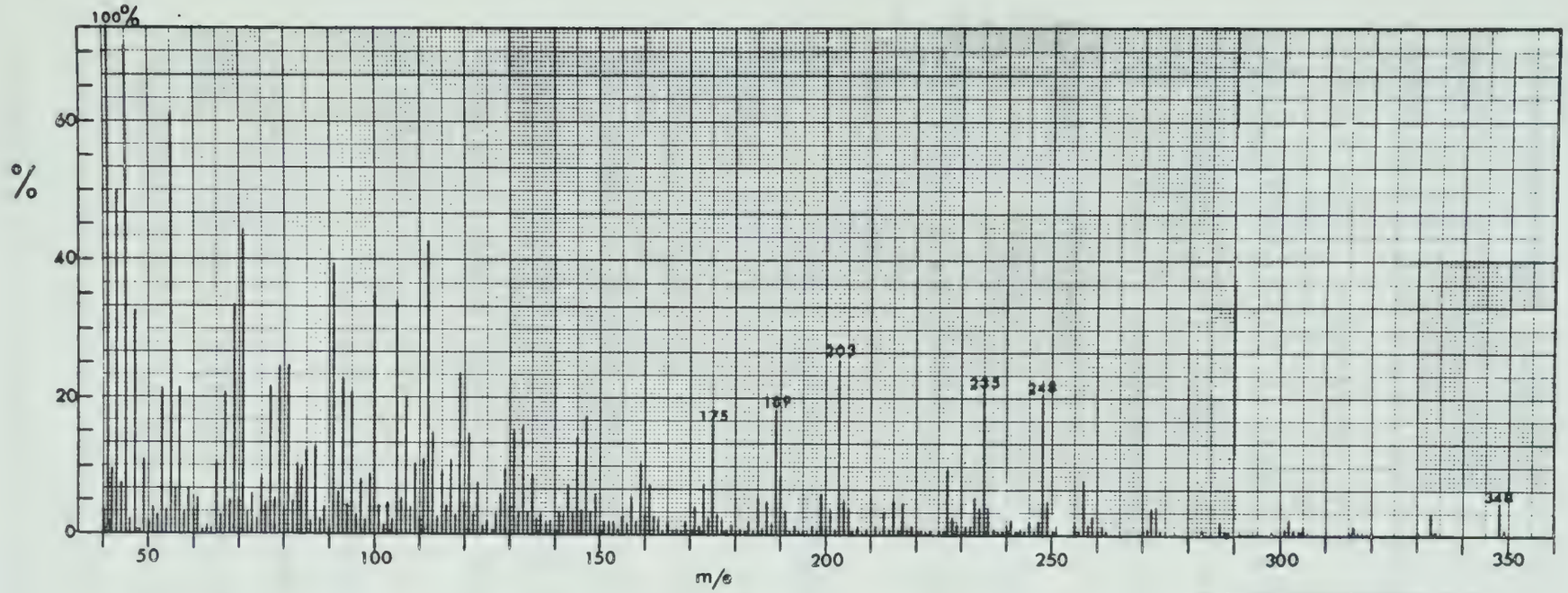
The infrared spectrum (figure 20) shows a

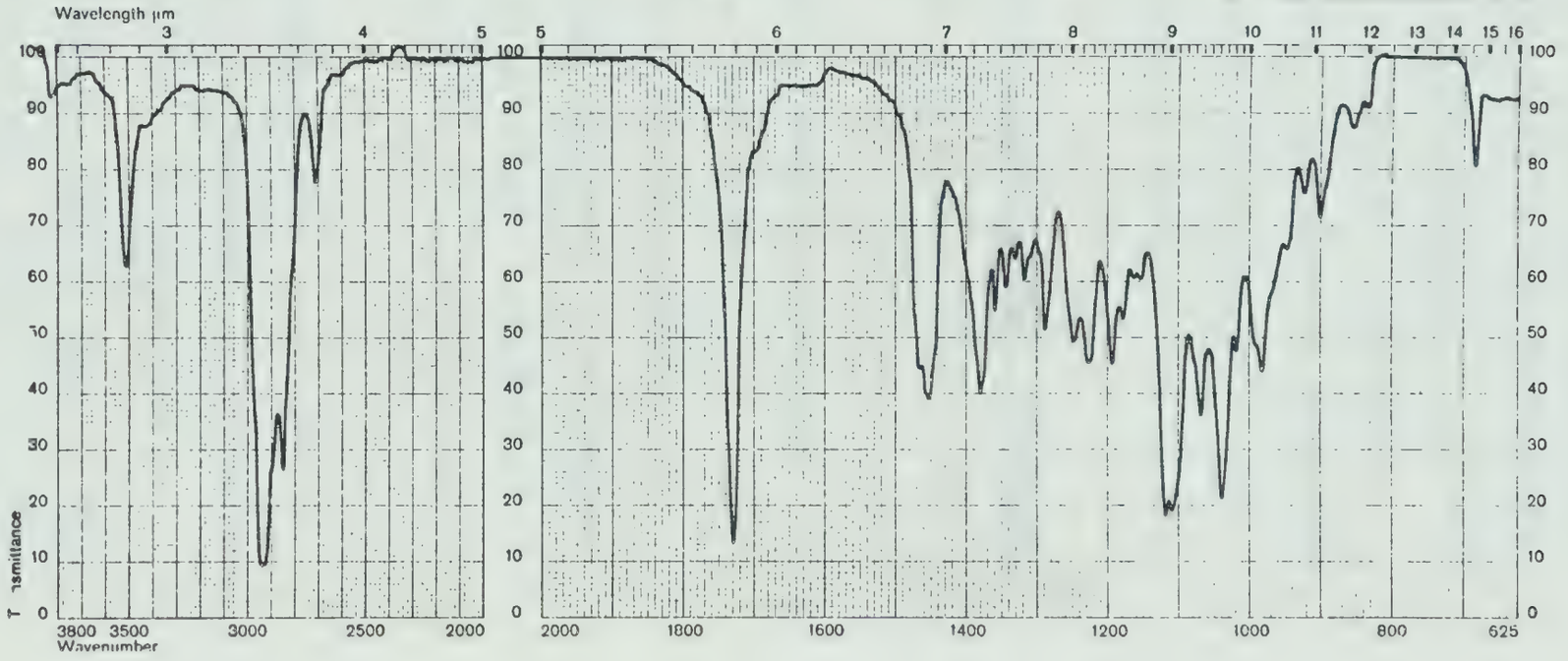
TOP: FIGURE 19

Mass spectrum of methanol adduct of cyathin B^3

BOTTOM: FIGURE 20

Infrared spectrum (CCl_4) of methanol adduct of cyathin B^3


100%
%
60
40
20
0
175
189
203
233
248
346
50
100
150
200
250
300
350
m/e


Wavelength μm
3
4
5
6
7
8
9
10
11
12
13
14
15
16
100
90
80
70
60
50
40
30
20
10
0
Transmittance
3800
3500
3000
2500
2000
Wavenumber
2000
1800
1600
1400
1200
1000
800
625

sharp band at 3518 cm^{-1}, indicative of an intramolecular hydrogen bonded hydroxyl. As a model, cis-cyclopentane-1,2-diol shows a sharp band in the infrared at 3572 cm^{-1} due to an intramolecular hydrogen bonded O-H stretching vibration (ref. 16, p. 183). Aldehydic absorption at 2715 and 1730 cm^{-1} indicates a saturated aldehyde. The normal intensity and presence of only one carbonyl band suggest that the molecule is in the hemi-ketal form.

The uv spectrum also supports the saturated aldehyde, with only a shoulder at 282 nm (ϵ=137) being present.

The nuclear magnetic resonance spectrum (Table V) of the material was very informative. The methoxyl singlet at δ3.36 varifies the addition of methanol. Since the hemi-ketal hydroxyl is hydrogen bonded as indicated by the ir spectrum, the Michael addition must have taken place to the α,β-unsaturated aldehyde at C-13.

The couplings between protons a, u, and b also confirm the stereochemistry depicted in structure 20. Proton a (δ4.12) appears as a doublet (J=4 Hz), and its low field chemical shift suggests that an oxygen occurs on the same carbon atom. Proton u (δ3.00) appears as a doublet of doublets (J=8,4 Hz). When the signal at δ4.12 was irradiated, the signal for proton u was observed to collapse to a doublet (J=8 Hz).

TABLE V NMR DATA FOR THE METHANOL ADDUCT OF CYATHIN B[3]

Signal	Shift of Adduct in CCl_4 (δ)	Multiplicity	Coupled with	Coupling Constants (Hz)
a	4.12	d	u	4
b	4.59	ddd	u,g,j	8,4,2.5
e	2.74	qq	s.t	7,7
f	2.3-2.0	(dd)	(q,j)	(?,?)
g	2.3-2.0	(ddd)	(j,f,b)	(?,?,4)
h,i	2.0-1.6	u	?	?
j	2.3-2.0	(ddd)	(g,f,b)	(?,?,2.5)
k,l,m n,o,p	1.6-1.2	u	?	?
q	0.92	s	----	----
r	0.92	s	----	----
s	0.92	d	e	7
t	0.82	d	e	7
u	3.00	dd	b,a	8,4
w	3.36	s	----	----
x	9.77	s	----	----

Proton b (δ4.59) appears as an eight line signal (doublet of doublet of doublets) with coupling constants of 8, 4, and 2.5 Hz. The coupling of 8 Hz with proton u was verified when the signal for proton u (δ3.00) was irradiated, causing proton b to collapse to a doublet of doublets (J=4, 2.5 Hz). These couplings of 4 and 2.5 Hz in the proton b signal are believed to be due to protons g and j absorbing in the complex region δ2.3-2.0.

When a model was constructed of structure 20, the dihedral angle between C_{13}-H_a and C_{12}-H_u was found to be approximately 120°. According to the Karplus rules[17], this dihedral angle should give a vicinal coupling constant of about 4 Hz between the two protons, which is observed in the spectrum of the methanol adduct. Also, the dihedral angle between C_{12}-H_u and C_{11}-H_b is approximately 15°. The coupling constant for these two protons is calculated to be about 7.5 Hz, agreeing very well with the observed 8 Hz. The coupling constant between the aldehydic proton and proton u must be very small, since it is not observable.

If methanol had added to C-12 as in the case of cyathin A[3] the nmr spectrum would be expected to have been quite different. The rest of the spectrum is consistent with the remainder of structure 20 for the methanol adduct. Attempts to crystallize the compound were unsuccessful.

Thus it has been shown that the α,β-unsaturated aldehyde of cyathin B^3 is very reactive toward Michael-type additions, and that its reactivity can be controlled by using the proper conditions.

Furthermore, the adduct was subjected to the normal acetylation procedure (acetic anhydride: pyridine, 1:2; at room temperature), but tlc indicated only starting material after a prolonged reaction time. When the reaction was carried out in refluxing pyridine and acetic anhydride, decomposition was observed by tlc and ir spectroscopy. Thus it seems that the alcohol group is quite inert to acetylation, attributed to the hydrogen-bonding with the methoxyl and the hemi-ketal formation.

4) Hydrogenation of Cyathin B^3-C^3 Mixture

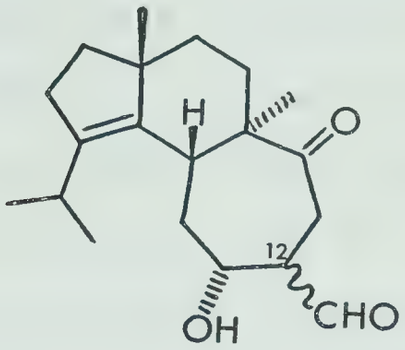
An experiment that was deemed useful was the catalytic hydrogenation of the cyathin B^3-C^3 mixture to one compound - dihydrocyathin B^3. From previous experience in these laboratories with allocyathin $B^{3\ 5}$, it was known that both the 1,2- and the 12,13-double bonds hydrogenated, while the tetrasubstituted 3,4-double bond remained unreactive. When the actual experiment was carried out, tlc indicated that hydrogenation had taken place since a spot of lower R_f than the starting material was present, and this compound could not be detected by the uv method (loss of the chromophore). Purification by preparative tlc lead to the formation of <u>two</u> spots when a subsequent analytical plate was run. Mass spectrometry gave the correct parent peak at m/e 318 and exact mass measurements of this peak revealed the formula $C_{20}H_{30}O_3$.

Infrared spectroscopy showed typical hydroxyl absorption at 3600 and 3400 cm^{-1}, along with characteristic aldehydic C-H stretch at 2800 and 2700 cm^{-1}. Carbonyl bands attributed to the aldehyde at 1728 cm^{-1} and the ketone at 1705 cm^{-1} were present. This latter band at 1705 cm^{-1} had only one-half the intensity of the 1728 cm^{-1} band, indicating that the ketone was in equilibrium with its closed, hemi-ketal form.

The nmr spectrum was very complex, and broad. Aldehyde absorption was shown by two signals at δ9.75

and 9.66. No olefinic absorption was observed.

The hydrogenated material is thus assigned to be a mixture of C-12 epimers of dihydrocyathin B^3 21.



21

The hydrogenation is assumed to have given the β epimer at C-12 since this would be the one predicted by steric arguments. When the material was chromatographed over silica gel, epimerization occured at C-12 to give a mixture of α and β epimers of dihydrocyathin B^3. Also the products seem to be in equilibrium with their hemi-ketal forms, as in the case of the starting material. Further production of dihydrocyathin B^3 and separation of the epimers was not attempted.

Isolation and Characterization of Palmitic Acid

On one occasion a material was isolated from a column chromatographic separation of crude cyathin which was eluted from the column before the cyathin B^3-C^3 band. This material crystallized after solvent removal and was found to be very soluble in hydrocarbon solvents, but only slightly soluble in 95% ethanol. Recrystallization from 95% ethanol gave colorless waxy-like crystals, m.p. 56-57°. Subsequent analytical tlc of this material showed it to be quite difficult to detect by the normal detection methods, but when a $Ce(SO_4)_2/H_2SO_4$ spray reagent was used, with subsequent heating, a brown spot was produced, R_f(A) 0.65.

Exact mass measurements of the parent peak at m/e 256 in the mass spectrum (figure 21) gave $C_{16}H_{32}O_2$ for the molecular formula.

The infrared spectrum (figure 22) shows broad absorption between 2500-3400 cm^{-1}, and a carbonyl band at 1720 cm^{-1}, indicative of a carboxylic acid.

At this point a structure can be suggested for this material. Since the molecular formula shows only one unsaturation, and this is in the form of a carboxyl group, then the compound must be a sixteen carbon fatty acid.

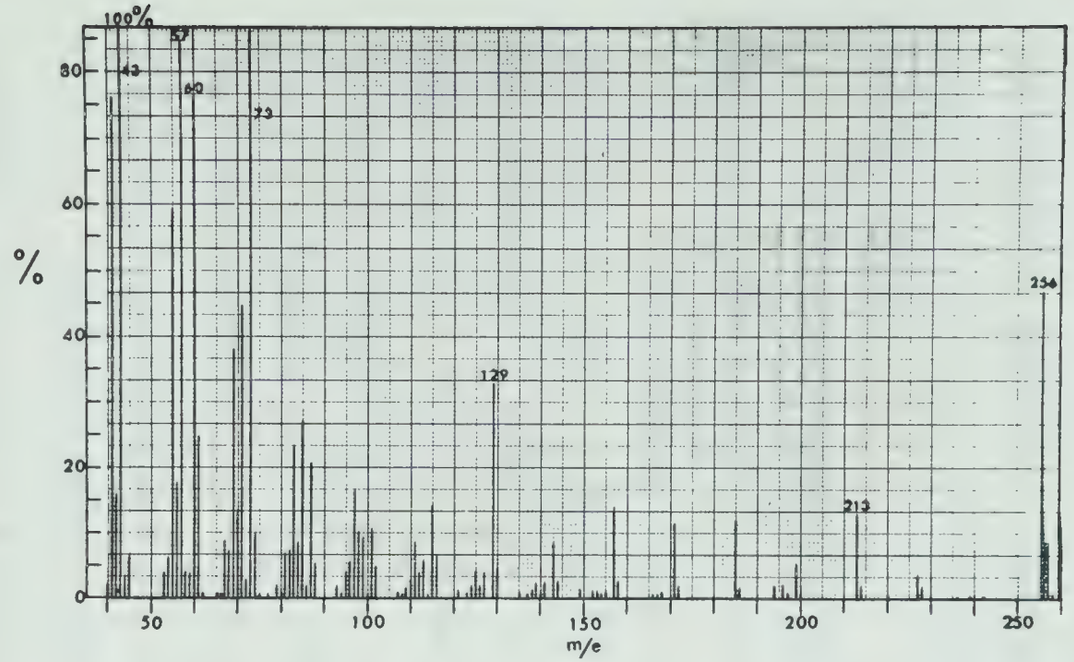
The nuclear magnetic resonance spectrum

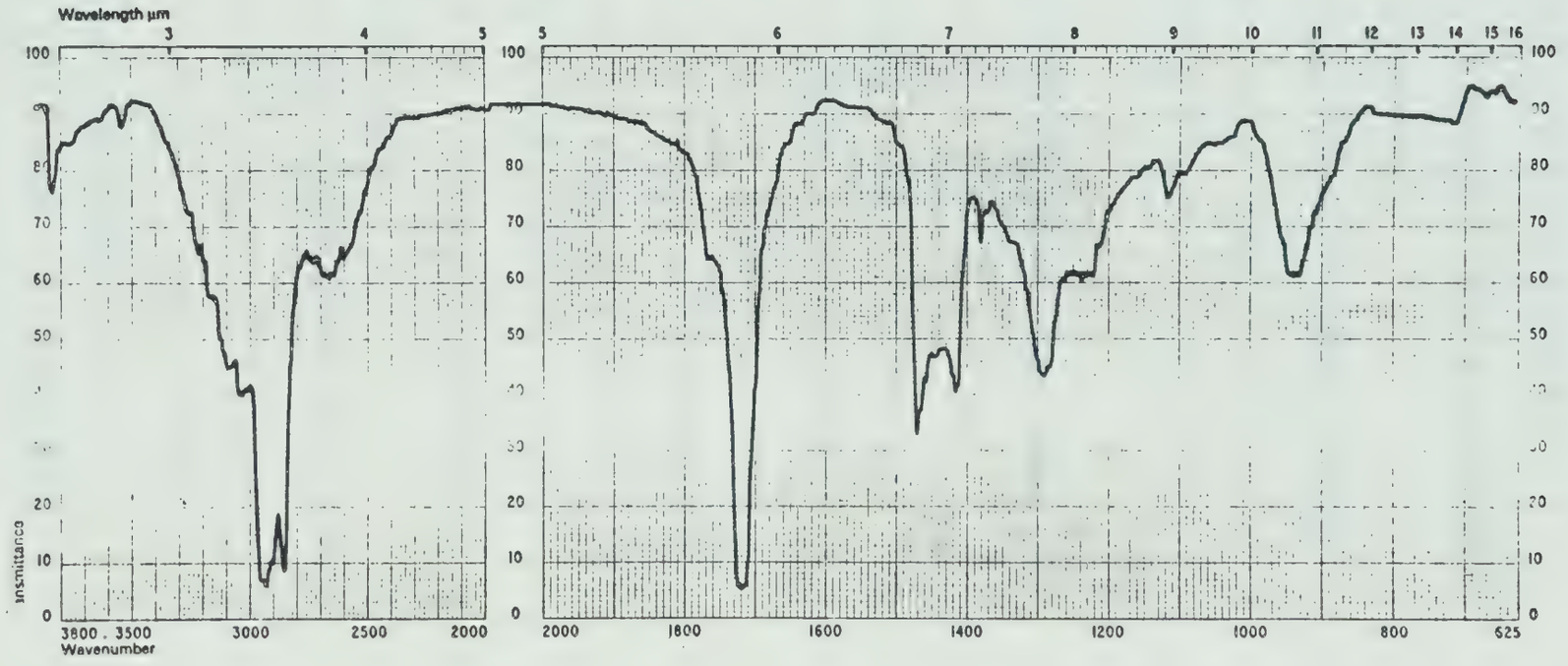
TOP: FIGURE 21

Mass spectrum of isolated palmitic acid

BOTTOM: FIGURE 22

Infrared spectrum (CCl_4) of isolated palmitic acid


100%
%
80
60
40
20
0
43
57
60
73
129
213
234
50
100
150
200
250
m/e


Wavelength µm
3
4
5
6
7
8
9
10
11
12
13
14
15
16
100
80
70
60
50
40
30
20
10
0
Transmittance
3800 . 3500
3000
2500
2000
Wavenumber
2000
1800
1600
1400
1200
1000
800
625

showed clearly this material to be the straight-chain sixteen carbon carboxylic acid - palmitic acid. Only one methyl group appears in the spectrum (δ0.88), as a triplet distorted by virtual coupling (see Experimental for remainder of the nmr spectrum).

When the melting point of an authentic sample of palmitic acid was determined, and compared with the isolated material, the following was found: m.p. 55-57°, m.m.p. 55-57°.

All of the spectral and physical data confirm that the material isolated is palmitic acid.

The compound was further characterized as its methyl ester, prepared by adding a solution of diazomethane in ether to the acid in ether solution. Mass spectral data confirmed the presence of the ester with the appearance of the parent peak at m/e 270. In the infrared spectrum, the broad hydroxyl was lost, and the carbonyl band shifted to 1750 cm^{-1}, confirming that esterfication had occurred.

At this point there was speculation that the palmitic acid was an artifact and not truly a fungal metabolite. The only source of palmitic acid if it was an artifact would have been the media used to grow the fungus. Therefore, one liter of the media was subjected to the normal extraction procedure. A small amount of material (24 mg) was obtained, but

tlc and mass spectrometry showed palmitic acid was not present. The majority of the material was grease and plasticizers, as judged by mass spectrometry.

Isolation of Cyathin A^2

During the earlier part of this work, crude metabolites obtained from a "normal" growth were subjected to preparative tlc separation. A band at R_f higher than cyathin B^3-C^3 was noted, and this band was removed, eluted, and subjected to the usual spectroscopic examination.

High resolution mass spectrometry indicated the molecular formula $C_{20}H_{30}O_2$ for the parent peak at m/e 302 (figure 23). According to the naming scheme of cyathins, this new material was designated as cyathin A^2.

The infrared spectrum (figure 24) shows broad absorption between 2500 - 3400 cm^{-1} and a strong carbonyl band at 1710 cm^{-1}, indicative of a carboxylic acid. Also, the uv spectrum showed a maxima at 213 nm (ϵ=7000) indicating the possibility that the acid could be α,β-unsaturated.

The nuclear magnetic resonance spectrum (figure 25) proves to be quite complex, but the broad signal at δ10.92 supports the carboxylic acid proposal.

Cyathin A^2 was further characterized to be a carboxylic acid by esterification with diazomethane in ether to produce a new compound of lower polarity on tlc. The mass spectral data (m/e 316) corresponded to $C_{21}H_{32}O_2$, and the infrared spectrum (no broad hydroxyl, 1730 cm^{-1}) suggests that an ester had been

TOP: FIGURE 23

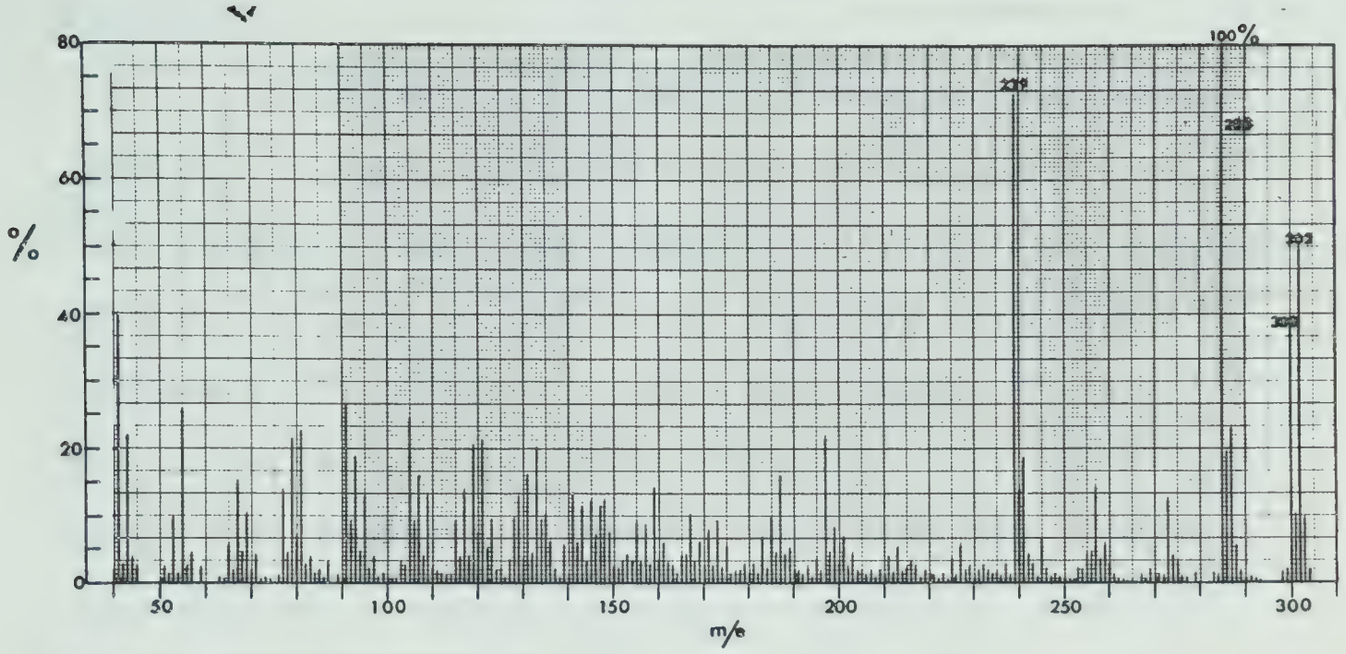
Mass spectrum of cyathin A^2

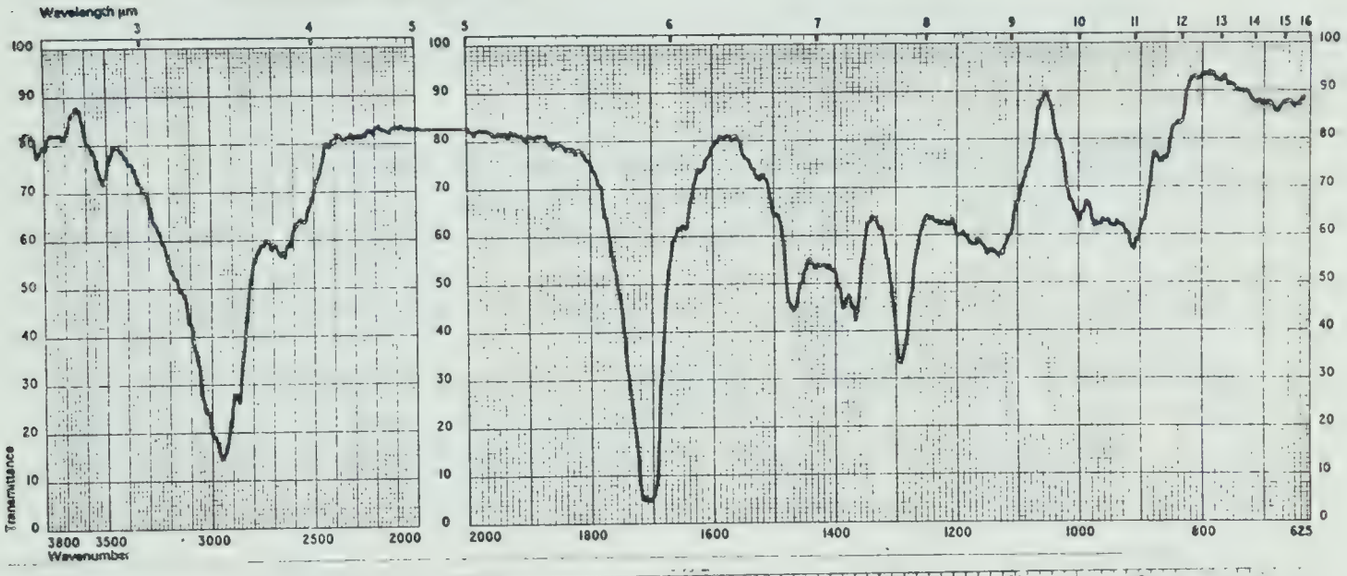
CENTER: FIGURE 24

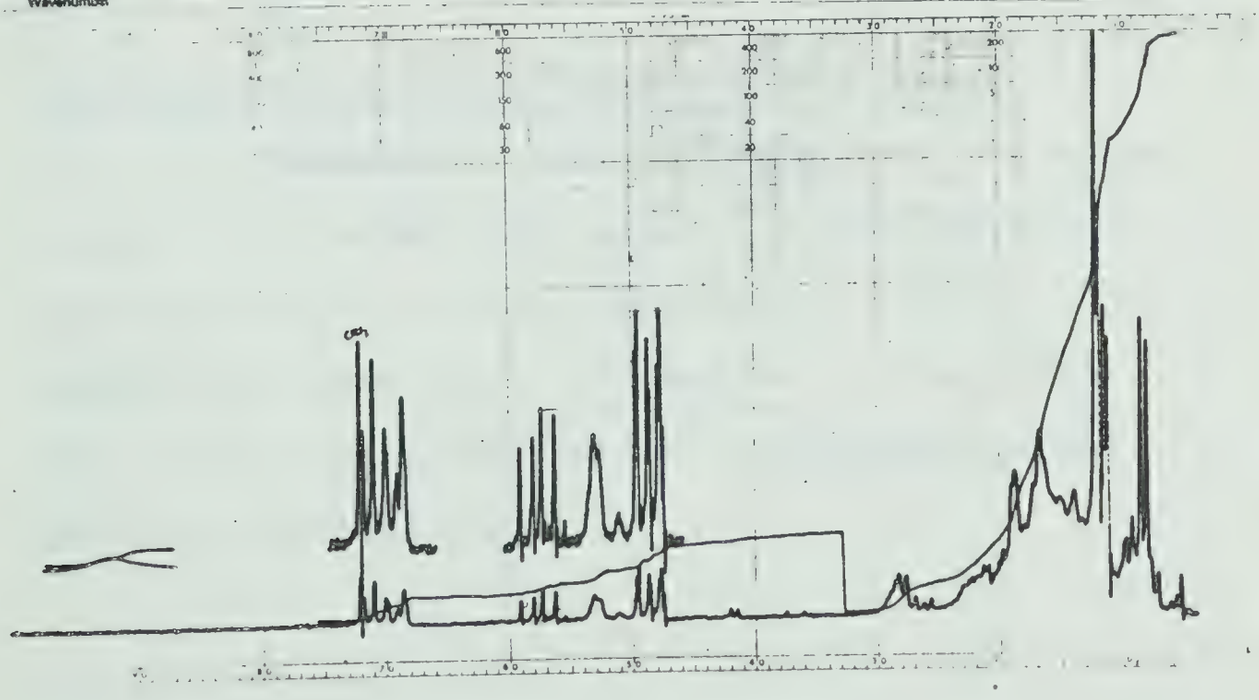
Infrared spectrum (CCl_4) of cyathin A^2

BOTTOM: FIGURE 25

Nuclear magnetic resonance spectrum ($CDCl_3$) of cyathin A^2


100%
%
m/e


Wavelength µm
Transmittance
Wavenumber



produced.

The peak at m/e 300 in the mass spectrum of cyathin A^2, along with the complexity of the nmr spectrum suggested that two compounds were present, one the dehydro analogue of the other, possibly analogous to cyathin B^3-C^3 chemistry. When the cyathin A^2 material was subjected to argentated silica gel tlc, indeed two spots were detected. Thus it seems that the cyathin A^2 material was actually a mixture containing cyathin A^2 and its dehydro analogue, given the tentative name cyathin B^2.

Cyathin A^2 has been isolated on one other occasion, but only in small quantity. Identification was established by tlc and mass spectrometry. Due to the small quantities of cyathin A^2 material available, further work in this area has not been attempted.

Biological Activity of Cyathin B^3-C^3 Mixture

Recently it has been shown that the mixed crystals of cyathin B^3- cyathin C^3 show significant antibiotic and especially antifungal activity. The results of these tests, carried out by the Smith, Klyne and French Laboratories in Philadelphia are given in the Appendix.

III. GENERAL EXPERIMENTAL

Solvents

The commonly used solvents (reagent grade) were found to contain silicone grease and phthalate plasticizers (by IR spectroscopy). The small scale of the experiments made these contaminates intolerable. Therefore, ethyl acetate, chloroform, methanol, acetone, pentane, Skelly B, methylene chloride, benzene, and pyridine were distilled prior to use. p-Dioxane was refluxed with fresh sodium metal and subsequently distilled. Reagent diethyl ether and iso-octane were used directly as obtained.

Thin-Layer Chromatography (tlc)

1. Preparation of thin-layer plates

Thin-layer plates were prepared according to the method described in Stahl's handbook (ref. 18, p. 27), using DESAGA equipment (West Germany).

Two adsorbants were used throughout this work: silica gel G (E. Merck) containing 1% electronic phosphor ($ZnSiO_4$, General Electric), referred to as ordinary silica gel and silica gel G impregnated with 10% silver nitrate (also containing 1% electronic phosphor), referred to as argentated silica gel. For an adsorbant layer of 0.05 x 20 x 100 cm, slurries were made of 50 g silica gel G and 100 ml H_2O (ordinary plates), or 45 g silica

gel G and 100 ml of 5% aqueous $AgNO_3$ (argentated silica plates). After spreading with the DESAGA equipment, the plates were allowed to air-dry for approximately 1hr. The plates were then activated at 110-120° for 1.5 - 2.0 hr, taken from the oven, and allowed to cool for at least 3 hr before use. In the case of argentated plates, care was taken to protect them from light since the resulting darkened plates made detection of spots or bands difficult.

The dimensions of the plates used for preparative work (ptlc) were either 20 x 100, 20 x 20, or 20 x 10 cm ; for analytical work, 20 x 20, 20 x 10, or 20 x 5 cm. The thickness of the adsorbant layers were approximately 0.5 mm. In order to follow the course of a reaction or a column chromatographic separation, microslides (25 x 75 or 50 x 75 mm) coated with ordinary silica gel G were used. These were prepared by spreading 60 at a time on templates to give an adsorbant thickness of approximately 0.2 mm.

2. Application of sample

In analytical work, the sample was applied with the aid of a capillary tube. In preparative work, the sample was applied manually with a micropipet (fitted with a rubber bulb) manufactured form disposable pipets. The sample was dissolved in an amount of solvent such that the viscosity of the solution was low enough to permit free flow when medium pressure was applied to the bulb, but high enough viscosity so that the solution

would stay in the pipet when no pressure was applied to the bulb. After a little practice, the sample could be applied in a thin, straight line across the bottom of the adsorbant. Approximately 200 mg of a sample was applied to a 20 x 100 cm plate.

3. Elution of the plates and detection of the spots or bands

After the solvent had evaporated from the applied spot or band, the plate was placed in a closed tank containing the developing solvent system. Forty-five to sixty minutes were needed for the elution (10 min for microslides), after which the plate was removed, and the solvent allowed to evaporate. In some cases, a double development method was used in which the plate was dried after the first elution, then subjected again to another elution. These instances are indicated with the R_f values.

In analytical work the spots were visualized by spraying with 30% H_2SO_4 and subsequent heating to ~110° and/or by viewing the plate under short wave (254 nm) uv light. Compounds with chromophores absorbing in the 250 nm region were found to be very sensitive to this latter method.

In preparative work, the uv method was used extensively. When a compound was encountered which had only a weak chromophore, an analytical plate was first developed, the spot detected by the H_2SO_4 method, and

results extrapolated to the preparative plate.

In using argentated plates, both the uv and H_2SO_4 methods proved satisfactory. In the case of palmitic acid, both the uv and H_2SO_4 methods were found to be very poor, but spraying with a 5% solution of $Ce(SO_4)_2$ in 30% aq. H_2SO_4 produced a dark spot, after heating to ~110°.

4. Recovery of material after preparative tlc

Bands containing the organic material as determined by the visualizing methods were outlined with a pointed glass rod. These bands were separately scraped off with the aid of a microslide, placed in small glass columns, and eluted with ether. Ether proved to be the most suitable since it was both polar enough and easily removed. Evaporation of the solvent on a Buchi rotovapor yielded the separated component.

5. Recording R_f values and solvent systems

R_f values are recorded in the form $R_f(X)n$. The letter X denotes the solvent system used for development; the letter n refers to the distance the spot moved/ the distance the solvent moved.
When argentated plates were used, the expression "$AgNO_3$" is added within the parenthesis.

Since the plates used were not standardized, the age of the plates, tank saturation, humidity, etc., could cause irreproducibility in R_f values (by as much as 0.05 units). Whenever possible a known sample was

developed alongside of the sample being investigated to reduce the possibility of error.

The following solvent systems were used in this work:

A:	benzene-acetone-acetic acid	75:25:1
B:	benzene-acetone-acetic acid	90:10:1
C:	chloroform (0.75% ethanol)	
D:	chloroform-methanol	98:2
E:	chloroform-methanol	95:5
F:	Skellysolve B-acetone	3:2
G:	Skellysolve B-acetone	3:1
H:	Skellysolve B-acetone	5:1
I:	Skellysolve B-acetone	7:1

Column Chromatography

1. Preparation of the column

Quartz columns (45 or 50 mm i.d.) were used in this work. Silicic acid (Mallinkrodt, 100 mesh) containing 1% electronic phosphor was used for the adsorbant, the ratio to sample weight being approximately 80:1, to give a column height of 20-25 cm.

The column was fitted with a glass wool plug and a 1 cm layer of purified sea sand was applied. A slurry of the adsorbant in $CHCl_3$ was then carefully poured into the column. The column was kept in a vertical position and tapped lightly to remove any air bubbles. A 1000 ml reservoir was attached and $CHCl_3$

was allowed to run through the column to aid in the settling of the adsorbant. The column was allowed to stand overnight before use.

2. Application of sample

In general the sample was made up as in the case of ptlc samples, except that the solvent was exclusively $CHCl_3$. Filtration was sometimes necessary to remove insoluble particles (i.e., chromocyathin). The reservoir was removed from the column and the solvent level was allowed to come just to the adsorbant level. Then, with the aid of a pipet, the sample was carefully dripped onto the surface of the adsorbant and allowed to soak in. Once all of the sample had been applied, more $CHCl_3$ was added, along with another 1 cm layer of sea sand. The reservoir was attached, filled, and elution begun.

3. Band detection and fraction collecting

Bands were detected by shining uv light (254 nm) upon the column (room lights off). For this detection system, the use of quartz columns is necessary.

Fractions were collected at 125 ml intervals, except where a detected band warranted a disruption of this procedure. Evaporation of the solvent in each fraction, weighing and analytical tlc gave an excellent indication of the progress of the separation.

Measurement and Recording of Spectra

Mass spectra reported in this thesis were measured on an A.E.I. model MS-9 mass spectrometer and are recorded as a percentage of the base peak. High resolution mass spectrometry (hrms) was used to determine all molecular formulas, since the small amounts of samples available in this work made combustion analysis impractical. Eventual correlation with known compounds validates this approach. A direct probe (70-200°) at an ionization potential of 70 eV was used.

Infrared (ir) spectra were recorded on a Perkin-Elmer model 421 dual grating spectrophotometer or a Unicam SP1000 infrared spectrophotometer.

Nuclear magnetic resonance (nmr) spectra were measured on a Varian Associates HR-100 spectrometer. Tetramethylsilane (TMS) was used as reference and lock signal, except when samples were run in benzene-d_6 in which TMS was the reference signal and benzene-d_6 the lock signal. The δ scale has been used throughout this thesis to express chemical shift values. The coupling pattern of signals is denoted by: s=singlet, d=doublet, t=triplet, q=quartet, m=unresolved multiplet, b=broad, and u=unresolved signal.

Ultraviolet (uv) spectra were recorded on a Cary Recording Spectrophotometer, model 15.

Circular dichroism (cd), and optical rotations ($[\alpha]_D$) were determined on a Durrum-Jasco Re-

cording Spectropolarimeter. The small amounts of samples used could bring about an error of as much as 50% in the extinction coefficient (ϵ), dichroic absorption ($\Delta\epsilon$), and optical rotation ($[\alpha]_D$).

Sample Preparation for Spectroscopic Measurements

To effectively remove traces of solvents, the sample was "purged" with the appropriate solvent. This was accomplished by dissolving the sample in a small volume of the solvent being used for the measurement, evaporationg under reduced pressure, and repeating this procedure several times. High vacuum drying used along with the purging method proved very effective.

Samples were submitted to the various Spectroscopic Services of this department for the measurement of mass, nmr, and some ir spectra. Most ir, and all uv, cd, and $[\alpha]_D$ measurements were carried out by the author.

Melting Point Apparatus

Melting point determinations were carried out on a Fisher-Johns melting point apparatus.

IV. DETAILED EXPERIMENTAL

Production and Isolation of Crude Cyathin

Cultures of the fungus Cyathus helenae were grown by a "still-surface" method in all of the work presented in this thesis. This method involved the use of a number of 2 liter Fernbach flasks (10-44), which were filled with 500 ml of defined liquid media (see Introduction for composition). After the flasks were autoclaved at 120°, 18 psi for 30 minutes, and allowed to cool to room temperature, they were innoculated with a fresh culture of the fungus. In the earlier part of this work, innoculation was accomplished by adding four 8 mm discs of the fungal mycelium taken from growths on Brodie's solid media (see Introduction for this composition also). In the latter part of this work a simpler method was devised in which a previous still-surface culture growing in a 500 ml Erlenmeyer flask on 300 ml of defined media was broken up with the aid of a sterile Waring blender, and pipeted into the flask to be innoculated by means of a sterile pipet.

After 25-30 days of growth the fungal broth was "harvested" by straining the contents of the flasks through cheesecloth, or in the latter part of this work (since Feb.,'73) by simply pouring off the liquid and leaving the mycelium behind in the

flasks. This latter method allowed easy regeneration of growth by simply adding fresh, sterile media to the flasks (this could be repeated 2-3 more times until the amount of mycelium became too great).

The brownish-colored broth was then extracted with an equal volume of ethyl acetate (extracted twice with half volumes of the solvent). The yellowish extract was then dried ($MgSO_4$), filtered, and evaporated to dryness to yield a yellow-brown foam. From 1 liter of broth there was obtained 0.2-0.5 g of crude cyathin.

Isolation of Cyathin B^3-C^3 Mixture: Column Chromatography

The column was prepared and the sample applied in the manner mentioned earlier (see General Experimental). The procedure for column elution was the same as previously described [4,5].

Example:

On September 13, 1972, 2.70 g of crude cyathin (from March, '70 growth) was chromatographed on 220 g silicic acid containing 1% electronic phosphor using a 50 mm diameter quartz column with chloroform as eluent. The 13^{th} 125 ml fraction (a strongly uv active band) crystallized after solvent removal, and was found to correspond to cyathin B^3-C^3 mixture. There was obtained 326 mg of crude cyathin B^3-C^3, R_f(A) 0.60. The other cyathin constituents were

obtained by continuing the elution procedure (grading into 2%, then 5% methanol in the chloroform).

Crystallization and Characterization of Cyathin B^3-C^3 Mixture

Cyathin B^3-C^3 mixture (326 mg) obtained as previously described, was recrystallized from ether-pentane. This was accomplished by first dissolving the sample in ether (~2 ml) in a 5 ml Erlenmeyer flask, filtering to remove solid particles (lint, silica, etc.), reducing the volume to ~1 ml, and adding pentane until the solution became cloudy. The addition of another drop of ether effected solution and the flask was allowed to stand at room temperature overnight. The crystals observed the next day were quite large (~0.5 x 0.5 x 2.0 mm). After another recrystallization colorless needles (130 mg) were obtained; m.p. 131-133°; hrms: calcd for $C_{20}H_{28}O_3$: 316.2038, found: 316.2037; uv (p-dioxane) ϵ_{233} 6800, ϵ_{350} 75 (shoulder). Mass, ir, and nmr spectra are reproduced in Chapter II.

Attempted $AgNO_3$-tlc Separation of Cyathin B^3-C^3 Mixture

Crystalline cyathin B^3-C^3 mixture (100 mg) was applied to four 10 x 20 cm plates coated with 10% $AgNO_3$/silica gel G. Development with solvent system F gave two slightly overlapping bands as detected by uv, $R_f(AgNO_3,F)$ 0.38 and 0.34. Three fractions were

removed (material between the two bands made up the second fraction). Elution with ether of the three fractions, evaporation of the solvent, and subsequent analytical tlc showed that impurities at both higher and lower R_f's were present in all three of the fractions. The main constituent of both fraction #1 (R_f 0.38) and fraction #2 was cyathin B^3 (83 mg) and fraction #3 (R_f 0.34) contained mostly cyathin C^3 (19 mg). Comparison of the m/e 316 and 314 peaks in the mass spectra lead to these assignments. A subsequent ptlc of the combined fractions #1 and #2 (on ordinary silica plates) produced crystals of cyathin B^3 (47 mg, m.p. 131-134°), still contaminated by cyathin C^3 (as determined by mass spectrometry, m/e 316 (68) and 314 (6)). The nmr spectrum also showed this material to still contain cyathin C^3.

Ketalization of Cyathin B^3-C^3 Mixture

Cyathin B^3-C^3 (77 mg) was dissolved in anhydrous methanol (2 ml) and a saturated solution of hydrogen chloride in methanol (1.5 ml) was added at room temperature. After 48 hr, tlc indicated complete reaction and the solvent was removed under reduced pressure. A non-crystalline mixture of cyathin B^3-C^3 methyl ketals (75 mg) was obtained R_f(G) 0.64, R_f($AgNO_3$,G) 0.62 and 0.55; hrms: calcd for $C_{21}H_{30}O_3$: 330.2195, found: 330.2184; Mass spectrum: 330(12), 328(30), 315(16), 313(8), 299(14), 295(10), 253(16), 225(11), 202(31), 189(53), 187(100),

175(14), 173(29), 161(20), 159(24), 145(30), 133(24), 131(18), 119(29), 105(26), 91(24), 55(26), 41(20); ir (CCl_4, cm^{-1}): 3060(w), 2820, 2720, 1695(s), 1625(w); nmr (CCl_4): δ 9.91(s,-CHO), 9.88(s,-CHO), 7.03(s,H-13), 6.97(s,H-13), 6.13(2d,H-1 and H-2, J=5.5,5.5), 5.03 (m, H-11), 3.27 (s,-OCH_3), 3.25 (s,-OCH_3), 2.92 (m,H-18), 2.1-2.5 (m), 1.7-1.3 (m), (1.10, 1.04, 0.97, 0.91, 0.86, 0.75 methyl region complex).

Argentated Silica Gel Ptlc of Cyathin B^3-C^3 Methyl Ketal Mixture

Cyathin B^3-C^3 methyl ketal mixture (75 mg) was applied to four 10 x 20 cm plates coated with 10% $AgNO_3$/ silica gel G. Development with solvent system G gave two bands. These were worked up in the usual manner. Cyathin B^3 methyl ketal (23 mg) gave the following properties: $R_f(AgNO_3,G)$ 0.62; $[\alpha]_D$ -105° (c, 0.1, iso-octane); hrms: calcd for $C_{21}H_{30}O_3$: 330.2195, found 330.2184; uv(iso-octane): ϵ_{233} 5300; cd (c, 0.1, iso-octane): $\Delta\epsilon_{346}$ -0.97, $\Delta\epsilon_{235}$ -2.78; Mass, ir, and nmr spectra or data are reproduced in Chapter II. Attempts to crystallize this material were unsuccessful.

Cyathin C^3 methyl ketal (16 mg) gave the following properties: $R_f(AgNo_3,G)$ 0.55; $[\alpha]_D$ -89° (c,0.043, iso-octane); hrms: calcd for $C_{21}H_{28}O_3$: 328.2039, found: 328.2042; uv (iso-octane): ϵ_{236} 7400, ϵ_{257} 4300(sh); cd (c, 0.043, iso-octane): $\Delta\epsilon_{356}$-1.23, $\Delta\epsilon_{258}$-4.31, $\Delta\epsilon_{235}$-9.23; mass, ir, and nmr spectra or data are reproduced in Chapter II.

Attempts to crystallize this material also met with failure.

Reduction of Cyathin B^3 Methyl Ketal

Cyathin B^3 methyl ketal (14 mg) was dissolved in 95% ethanol (0.5 ml), and 0.5 ml of a solution of sodium borohydride in ethanol (1 mg/ml) was added. After 15 min tlc indicated complete reduction. The solvent was removed under reduced pressure, water added, and the solution extracted with carbon tetrachloride. Drying of the carbon tetrachloride layer, followed by evaporation gave a material (10 mg) which was slightly impure as indicated by tlc. Purification by ptlc gave an oil (4 mg) identical with cyathin A^3 methyl ketal: R_f(G) 0.31 (not uv detectable); $[\alpha]_D$ -158° (c, 0.06, iso-octane) ; hrms: calcd for $C_{21}H_{32}O_3$: 332.2352, found:332.2346; uv(iso-octane) end absorption only; cd (c, 0.06, iso-octane): $\Delta\epsilon_{300}$ 0.0 ; mass, ir, and nmr spectra or data are reproduced in Chapter II.

Reduction of Cyathin C^3 Methyl Ketal

Cyathin C^3 methyl ketal (22 mg) was dissolved in 95% ethanol (0.5 ml) and 0.5 ml of a solution of sodium borohydride in ethanol (1 mg/ml) was added. After 15 min tlc indicated complete reduction; the solvent was removed under reduced pressure, water added, and the solution extracted with carbon tetrachloride. There was obtained a material (20 mg) whose properties were consistent with those of allocyathin B^3 methyl ketal: R_f(G) 0.38;

$[\alpha]_D$ -179° (c, 0.067, iso-octane); hrms: calcd for $C_{21}H_{30}O_3$: 330.2195, found: 330.2194; uv (iso-octane): ϵ_{255} 3500; cd (c, 0.067, iso-octane): $\Delta\epsilon_{253}$ -3.46; mass ir, and nmr spectra or data are reproduced in Chapter II.

Acetylation of Cyathin B^3-C^3 Mixture

Cyathin B^3-C^3 mixture (20 mg) was acetylated for 8 hrs at 40° in methylene chloride (1 ml) containing pyridine (0.16 ml) and acetic anhydride (0.08 ml). The solution was then evaporated and purged twice with benzene, washed with H_2O, dried, and the benzene evaporated. The crude product (21 mg) was chromatographed by ptlc (solvent system H, double elution), yielding 4 overlapping bands. The least polar compound (5 mg), R_f(H, double elution) 0.65, showed no acetate or hydroxyl absorption in the infrared and an apparent molecular ion at m/e 298 in the mass spectrum (base peak at m/e 283). This compound is described in the next section.

The two intermediate bands (7 mg), R_f(H, double elution) 0.56-0.60, showed properties consistent with those expected for O-acetylcyathin B^3: mass spectrum: m/e 358(9.5), 343(10), 316(19), 298(18), 283(28), 255(26), 222(22), 189(29), 199(29), 105(38), 91(43), 55(47), 43(100), 41(62); ir (CCl_4): no hydroxyl, 2810, 2710, 1745, 1710, 1680 cm^{-1}.

The compound with the highest polarity (2 mg), R_f (H, double elution) 0.55, appeared to consist of mainly O-acetylcyathin C^3, along with some O-acetylcyathin B^3:

mass spectrum: m/e [358(5)], 356(11), 343(5), 341(2), 315(4), 314(3), 298(3.5), 296(3.5), 283(4.5), 281(3.5), 253(9.5), 225(7.5), 201(10.5) 187(10), 119(21), 105(22), 91(31), 55(40), 43(100), 41(56); ir(CCl_4): no hydroxyl, 2720, 1750, 1705, 1690 cm^{-1}.

Dehydration of Cyathin B^3-C^3 Mixture via Acetylation

Cyathin B^3-C^3 (168 mg) was dissolved in methylene chloride (8 ml) containing acetic anhydride (0.8 ml) and pyridine (1.6 ml). After 71/2 hr at 50°, tlc indicated only two components. After work-up in the usual manner, the crude product was subjected to ptlc (solvent system I, double elution).

The compound of lowest polarity (81 mg) showed properties consistent with anhydrocyathin B^3: R_f(I, double elution) 0.54; $[\alpha]_D$ +158° (c,0.021, iso-octane); hrms: calcd for $C_{20}H_{26}O_2$: 298.1933, found: 298.1937; uv (iso-octane): ϵ_{259} 6900, ϵ_{325} 12,100; cd (c, 0.021, iso-octane): $\Delta\epsilon_{373}$-1.40, $\Delta\epsilon_{324}$+12.1, $\Delta\epsilon_{258}$-4.75, $\Delta\epsilon_{238}$+6.05, $\Delta\epsilon_{214}$+0.43; mass, ir, and nmr spectra or data are reproduced in Chapter II. Anhydrocyathin B^3 was obtained as a light yellow oil and has not yielded to crystallization attempts.

The compound of slightly greater polarity (42 mg) crystallized in the absence of solvent after being allowed to stand in the fridge for a week. Recrystallization was effected with ether-pentane; light

yellow needles, m.p. 105.5-107°. The following properties are consistent with anhydrocyathin C^3: R_f(I, double elution) 0.48; $[\alpha]_D$ -33° (c, 0.014, iso-octane); hrms: calcd for $C_{20}H_{24}O_2$: 296.1776, found: 296.1774; uv(iso-octane): ϵ_{264} 6000, ϵ_{345} 10,400; cd (c, 0.014, iso-octane): $\Delta\epsilon_{377}$-0.63, $\Delta\epsilon_{371}$+5.7, $\Delta\epsilon_{286}$-1.3, $\Delta\epsilon_{270}$+1.0, $\Delta\epsilon_{263}$+0.60, $\Delta\epsilon_{241}$+3.0, $\Delta\epsilon_{215}$-9.9; mass, ir, and nmr spectra or data are reproduced in Chapter II.

Methanol Adduct of Cyathin B^3-C^3 Mixture

Cyathin B^3-C^3 (20 mg) was dissolved in methanol (20 mls) and stirred under N_2 for 15 min to remove the oxygen dissolved in the solvent. Potassium carbonate (~2 mg) was added, and the solution stirred under N_2 for 10 min. Then 2 drops of 10% HCl were added to neutralize the base, 5% palladium on charcoal (40 mg) was added, and the solution hydrogenated under a H_2 atmosphere for 17 min at room temperature. The catalyst was filtered off, solvent evaporated, the residue dissolved in ether, filtered again, and the ether evaporated. A clear oil was obtained (20 mg) which gave the following properties: R_f(G) 0.38 (not uv detectable); $[\alpha]_D$ -62° (c, 0.16, methanol); hrms: calcd for $C_{21}H_{32}O_4$: 348.2301, found 348.2309; uv (MeOH): ϵ_{282} 137 (shoulder); cd (c, 0.16, methanol): $\Delta\epsilon_{296}$-0.047, $\Delta\epsilon_{255}$+0.045; mass, ir, and nmr spectra or data are reproduced in Chapter II.

Attempts to acetylate this compound at room

temperature in acetic anhydride/pyridine, 1:2, gave back starting material after 1 day. Acetylation with refluxing acetic anhydride/pyridine, 1:2, led to decomposition after 20 hrs as indicated by tlc and ir spectroscopy.

Hydrogenation of Cyathin B^3-C^3 Mixture

Cyathin B^3-C^3 (11 mg) dissolved in methanol (20 ml) was stirred under an atmosphere of H_2 for 17 min in the presence of 5% palladium on charcoal (22 mg). Filtration and evaporation of the solvent gave a clear oil (11 mg) which showed one major spot on tlc, R_f(G) 0.30, along with some minor impurities. This material could not be detected by the uv method on tlc plates. Infrared spectrum: 3580 and 3400 cm^{-1} (broad, hydroxyl), 2690 and 2790 cm^{-1} (aldehyde C-H), 1725 cm^{-1} (aldehyde C=O), and 1703 cm^{-1} (ketone C=O, partially masked).

Ptlc purification of this material (together with that of a subsequent hydrogenation, total 61 mg) yielded a material (38 mg) which now appeared as two spots on tlc, R_f(G) 0.33 and 0.41; hrms: calcd for $C_{20}H_{30}O_3$: 318.2195, found 318.2208; mass spectrum: m/e 318(62), 303(93), 300(25), 285(37), 275(16), 261(26), 257(22), 201(15), 190(100), 189(26), 175(60), 147(24), 119(24), 91(35), 69(31), 55(55), 41(78); ir (CCl_4): 3600 and 3400 (hydroxyl), 2800 and 2700 (aldehyde C-H), 1728 (aldehyde C=O), and 1705 (ketone C=O, partially masked); nmr (CCl_4): δ 9.75 and 9.66 (s, aldehyde

protons), no olefinic protons, the remainder of the spectrum was complex and broad.

Isolation, Crystallization, and Characterization of Palmitic Acid

Palmitic acid (n-$C_{15}H_{31}COOH$) was isolated from the fungal broth only once. On Dec. 11, 1972, 3.0g of crude cyathin (from a "normal" growth during July, 1972) was subjected to column chromatography. The 4^{th}-7^{th} 125 ml fractions contained a new component which crystallized after solvent removal (cyathin B^3-C^3 was also obtained, fractions 8, 9, and 10). This material (150 mg) was recrystallized from 95% ethanol to give colorless, waxy-like crystals, m.p. 56-57°, which showed the following properties: R_f(A) 0.65 (difficult to detect by both uv and H_2SO_4 methods); hrms: calcd for $C_{16}H_{32}O_2$: 256.2402, found: 256.2402; uv (p-dioxane): end absorption only; nmr ($CDCl_3$): δ2.34 (t, methylene α to carbonyl), 1.63 (broad t, methylene β to carbonyl), 1.29 (s, 24 hydrogens), 0.88 (broad t, methyl), carboxyl proton not detected. Mass, and ir spectra are reproduced in Chapter II.

An authentic sample of palmitic acid gave m.p. 55-57°, m.m.p. with the material isolated: 55-57°.

The isolated material was further characterized as its methyl ester (prepared by adding a 5 ml solution of diazomethane in ether to a solution of 20 mg substrate

in ether): mass spectrum: 270(55), 239(9), 227(10), 143(16), 87(88), 74(100), 69(23), 55(37), 43(47), 41(40); ir (CCl_4): no broad OH, 1750 cm^{-1}.

In order to show that palmitic acid was a fungal metabolite, and not an artifact from the media, 1 liter of defined media was extracted with ethyl acetate in the normal manner. A brown oil (24 mg) was obtained, which on tlc showed no spot corresponding to palmitic acid (detected by spraying with $Ce(SO_4)_2/H_2SO_4$ and heating, with which palmitic acid gives a dark spot). The mass spectrum of this "extract" showed the phthalate peaks (m/e 149, 279) to be predominant and m/e 256 to be very small.

Isolation of Cyathin A^2

Cyathin A^2 has been isolated only on two occasions, both close to the beginning of this work. On Feb. 10, 1972, a crude extract (0.67 g), from a growth the previous month, was subjected to ptlc. Five fractions were removed, and the first (least polar, R_f(A) 0.85) proved to be phthalate impurity by ir spectroscopy. The second was a new compound (106 mg), which is now called cyathin A^2 and gave the following properties: R_f(A) 0.75; hrms: calcd for $C_{20}H_{30}O_2$: 302.2246, found 302.2233; uv(cyclohexane): ϵ_{213} 7000; mass, ir, and nmr spectra are reproduced in Chapter II. The remaining three bands from the ptlc separation contained the other known

cyathins (A^3,A^4, etc.).

Since the spectral data of cyathin A^2 showed it to be a carboxylic acid, possibly an α,β-unsaturated acid, a small amount (15 mg) in ether was treated with a solution of diazomethane in ether (5 mls). After allowing the solution to stand overnight in the fridge, the ether was evaporated to give a material consistent with the methyl ester of cyathin A^2: hrms: calcd for $C_{21}H_{32}O_2$: 316.2402(nearest), found: 316.2996; ir ($CHCl_3$): no broad OH, 1730 cm^{-1}(ester); nmr ($CDCl_3$): same as cyathin A^2, except no signal at δ11.0 and a new signal at 3.64 (s, methyl ester).

Since the mass spectrum of cyathin A^2 shows a prominent peak at m/e 300 and since the nmr spectrum was so complex, it seemed that there were two compounds, differing only by two mass units present in the cyathin A^2 material. This was verified when cyathin A^2 was subjected to an argentated tlc plate. Two spots were observed (by spraying with 0.1% 2',7'-dichlorofluoresceine in EtOH and viewing under long wave uv light): R_f(A)0.70 and 0.64. Lack of material has precluded any further work on cyathin A^2.

REFERENCES

1. H. J. Brodie, Can. J. Bot., 44, 1235, (1966).
2. A. Olchowecki, M.Sc. thesis, Department of Botany, University of Alberta, 1967.
3. B.N. Johri, Ph.D. thesis, Department of Botany, University of Alberta, 1969.
4. A. D. Allbutt, W. A. Ayer, H. J. Brodie, B. N. Johri, and H. Taube, Can. J. Microbiol., 17, 1401 (1971).
5. H. Taube, Ph.D. thesis, Department of Chemistry, University of Alberta, 1972.
6. W. A. Ayer, and H. Taube, Tet. Lett., No. 19, 1917 (1972).
7. M. J. Bennett and R. M. Tuggle, submitted for publication.
8. J. M. Bobbitt, "Thin-Layer Chromatography", Reinhold, New York, 1963.
9. O. L. Chapman, J. Am. Chem. Soc., 85, 2014 (1963).
10. R. M. Silverstein, and G. C. Basler, "Spectrometric Identification of Organic Compounds", Second Ed., Wiley, New York, 1967.
11. A. S. Gupta, and S. Dev., J. Chromatog., 12, 189 (1963).
12. R. L. Augustine, "Reduction, Techniques and Applications in Organic Synthesis", Dekker, New York, 1968.
13. W. A. Ayer, and H. Taube, Can. J. Chem., 51, 3842 (1973).

14. H. O. Huisman, A. Smit, P. H. VanLeeuwen, and J. H. VanRij, Rec. Trav. Chim., 75, 977 (1956),

15. A. I. Scott, "Interpretation of the Ultraviolet Spectra of Natural Products", Macmillan, New York, 1964, p. 58.

16. C. N. R. Rao, "Chemical Applications of Infrared Spectroscopy", Academic Press, New York, 1963.

17. M. Karplus, J. Chem. Phys., 30, 11 (1959).

18. E. Stahl, Ed., "Thin-Layer Chromatography", Springer-Verlag, Berlin, 1965.

19. R. L. Augustine, "Catalytic Hydrogenation, Techniques and Applications in Organic Synthesis", Dekker, New York, 1965.

APPENDIX

July 29, 1974

MEMO TO: Dr. J. Weisbach

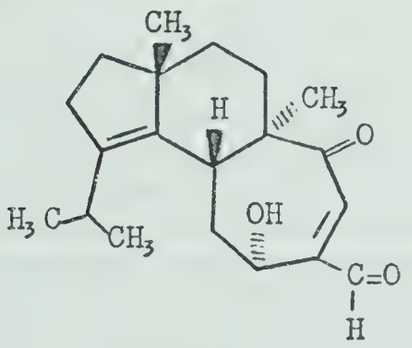
cc: Dr. Actor
Dr. Elander
Dr. Lourie
Dr. Misher
R&D Documents
Dr. Cramer

FROM: J. Uri/J. Guarini/M. Knight

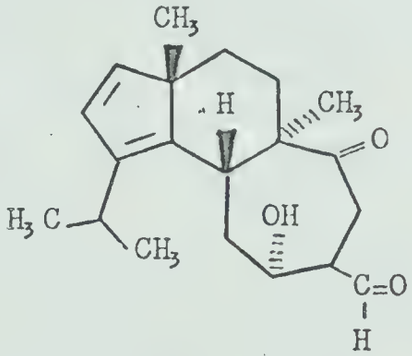
SUBJECT: In-Vitro Antimicrobial Spectrum of SKF 74227 (Cyathin B_3 - Cyathin C_3 Mixed Crystals).

Summary

This mixture of crystals, obtained from Dr. Ayer - University of Alberta, was found to have significant in-vitro activity against the Trichophyton mentagrophytes strain (BC-1258) and also good activity against the gram-positive cocci in the assay. Activity against the test gram-negative bacilla and Candida albicans is negligible or non-existant.



B_3



C_3

Methods and Materials

The conventional agar dilution method was used throughout the experiment. Dilutions (two-fold levels from 1000-0.5 µg/ml) of the compound mixture under test (SKF 74227) were mixed into the melted and cooled to 50°C Penassay Seed Agar, buffered to pH 7.0 by the addition of 10% McIlvain's citric acid-phosphate buffer. After the agar had hardened and dried in the petri dishes, appropriately diluted suspensions of the test microorganisms were applied to its surface using the Steers' multiple inocula replicating device. After overnight incubation at 37°C for bacteria and Candida albicans, and an additional 3-day incubation at 30°C for Trichophyton mentagrophytes, the individual median inhibitory concentration (MIC) values were read and recorded.

The distribution of the 15 test microorganisms was as follows: (a) gram-positive cocci (Nos. 1-3); (b) apathogenic mycobacterium (No. 4); (c) gram-negative bacilli (Nos. 5-13); (d) the yeast-like Candida albicans (No. 14) and (e) the dermatophyte, Trichophyton mentagrophytes (No. 15).

In addition to the compound (SKF 74227 or cyathin B_3 - cyathin C_3, mixed crystals) under test, three known antibiotics (gentamicin, erythromycin and amphotericin B) were included as controls. Their concentrations used in this experiment were from 200 to 0.1 µg/ml, also in two-fold dilutions.

Results and Discussion

SKF No. 74227 (Cyathin B_3 - Cyathin C_3, mixed crystals) when assayed in an in-vitro system showed interesting effect against the filamentous dermatophyte, Trichophyton mentagrophytes BC-1258 with an activity comparable to that of amphotericin B, but not against the yeast-like Candida albicans. (Both fungus strains are pathogenic to man.)

The test material also has appreciable but not great MIC values against both the penicillin-(No. 1) and erythromycin-(No. 2) resistant Staphylococcus aureus strains as well as the Streptococcus faecalis (Enterococcus) strain (No. 3). Activities against the gram-negative bacilli are insignificant.

The in-vitro activity of the mixture against the Trichophyton mentagrophytes strain should be considered interesting; therefore, it would be important to examine the activity of the mixture and the individual components against other dermatophyte strains, particularly against T. rubrum strains, which are not sensitive to tolnaftate (Tinactin). If SKF 74227 or either of its individual components proves to be active against these recently more and more frequently occurring pathogenic dermatophytes and is without tissue irritation, it may be a useful topical agent. In addition to this proposed in-vitro anti-dermatophyte expanded spectrum assay, its general and local toxicity data can supply information about its further and broader usefulness. For proposed further study additional supply (at least 1 gram) would be needed initially.

Notebook Ref.: 7726, p. 163

JU/JG MK/cdg

Table 1: In-Vitro Activities of SKF 74227 and Three Control Antibiotics

PENASSAY SEED AGAR (pH 7.0)		MIC (µg/ml)														
CODE	SK&F NO.	1 Staph. aureus HH 127	2 Staph. aureus SA 910	3 Strep. faecalis HH 34358	4 Mycobacterium phlei 1228	5 Proteus mirabilis PM 444	6 E. coli SKF 12140	7 Kleb. pneumoniae SKF 4200	8 Salmonella gallinarum ATCC 9184	9 Pseudomonas aeruginosa HH 63	10 Serratia marcescens ATCC 13880	11 Proteus morgani P-179	12 Providencia sp. PR-276	13 Enterobacter cloacae HH 31254	14 Candida albicans BC 759	15 Trichophyton mentagrophytes BC 1258
JRM-1	74227	2	2	4	16	1000	>1000	63	63	>1000	>1000	63	63	>1000	63	1
Gentamicin		1.6	6.3	25	1.6	6.3	1.6	0.8	6.3	1.6	6.3	1.6	12.5	1.6	>200	>200
Erythromycin		0.4	>200	>200	0.2	>200	50	100	200	200	100	50	200	100	>200	>200
Amphotericin-B		>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	0.4	0.8